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Abstract

Title of Dissertation:

Structure Function Analysis of the Ferric Uptake Regulator (Fur) of *Helicobacter pylori*

Beth M. Carpenter, Doctor of Philosophy, 2010

Thesis directed by:

D. Scott Merrell, Ph.D.
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Helicobacter pylori is a Gram negative, microaerophilic, spiral shaped bacterium that is the causative agent of gastritis and peptic ulcer disease and is implicated in the development of both mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma. *H. pylori* expresses several factors that aid in its colonization of and persistence within the harsh environment of the stomach. Among these factors is the Ferric Uptake Regulator (Fur). Fur traditionally represses genes by binding to select regions within target promoters (Fur boxes) in its iron-bound dimeric form, which results in the occlusion of the RNA polymerase binding site. This type of iron-bound Fur repression is utilized in *H. pylori* as well as widely in the bacterial world. Furthermore, *H. pylori* Fur has additionally been found to repress another set of genes in the absence of its iron co-factor in what is termed *apo*-Fur repression. As yet, *apo*-Fur repression has only been shown to occur in *H. pylori*, which makes the study of Fur in *H. pylori* particularly interesting. A plasmid- based transcriptional reporter system was developed

to monitor the expression of both iron-bound and *apo*-Fur regulated genes under iron-replete and iron-limited conditions and for use as a complementation vector. *fur* expressed on this plasmid was able to complement both forms of Fur regulation in a chromosomal *fur* deletion strain. *H. pylori* is a highly pleomorphic organism and gene variation between strains is a common phenomenon. It was observed that *apo*-Fur-dependent regulation of the *sodB* gene, which encodes *H. pylori*'s only superoxide dismutase protein, was not conserved in strain G27 as compared to strain 26695. This difference in regulation was determined to be the result of a single DNA base change within the Fur box region of the *sodB* promoter. This is the first residue that has been shown to be important for *apo*-Fur regulation in *H. pylori*. Lastly, because little is known about what amino acid (AA) residues are important for Fur regulation in *H. pylori*, six AA residues that are broadly conserved among Fur proteins were changed to an Ala in *H. pylori* Fur and then assessed for their role in Fur function. One residue, V64 did not alter Fur regulation while the remaining five AA residues exhibited a phenotype for Fur regulation. H96, E110, and E117 altered iron-bound Fur regulation while E90 and H134 altered *apo*-Fur regulation. Further analysis of these mutant Fur proteins revealed that H96 is important for iron binding and oligomerization, E117 is involved in iron binding, and E110 plays a role in DNA binding, iron binding, and oligomerization. In addition, H134 plays a role in DNA binding while E90 did not affect any of the studied aspects of Fur function. Taken together, the data presented in this thesis begin to unravel the complexity of Fur regulation in *H. pylori* and lay the foundation for continued study of this important regulatory protein.

Structure Function Analysis of the Ferric Uptake Regulator (Fur) of *Helicobacter pylori*

By

Beth Marie Carpenter

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Lastly, to my husband, Jon – This is as much a testament to your dedication as it is to mine. Thank-you - for everything. You have been my sanity and touchstone throughout graduate school, and I would not be so successful without you by my side. I love you!

Dedication

To the good God who saw fit to give humankind both heart and mind to know Him and His creation. May this work be pleasing unto You.

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Chapter One

Introduction

Helicobacter pylori

The organism

Helicobacter pylori is a bacterium with a fortunate history and a revolutionary impact on the medical world. Reports of spiral shaped bacteria in association with the gastric mucosa date as far back as the 1870s and are found sporadically throughout the early 20th century (as reviewed in (89)); however, the presence of these bacteria was largely disregarded as the organisms appeared to be unculturable. Then in the early 1980s, Warren and Marshall attempted to culture these bacteria from gastric biopsy specimens using *Campylobacter* culturing techniques. They were successfully able to do so after an accidental extended incubation of the culture plates (five days instead of the usual three) (140, 202). This organism was originally named *Campylobacter pyloridis* due to similarities with the *Campylobacter* genus. However, as this bacterium was more thoroughly studied, it was subsequently given its own genus and renamed *Helicobacter pylori* in 1989 (1). Currently, there are 18 members of this genus (160, 182) of which *H. pylori* is the type species. *Helicobacter* species have been found to infect the gastric mucosa, intestinal tract, and even the hepatobilliary tract in a wide range of mammals - from cats and dogs, to cattle and swine and even rodents (182). *H. pylori* naturally infects humans and some non-human primates (60-61, 112), and it is thought to have been co-evolving with humans for a minimum of 50,000 years (9, 45).

H. pylori is a small, Gram negative, microaerophilic, spiral shaped bacterium whose niche is the gastric mucosa. This bacterium has four to six polar, sheathed flagella (86, 90) and exhibits corkscrew motility (Figure 1). Several strains of *H. pylori* have been sequenced each revealing A/T-rich (60%) genomes of approximately 1.6Mb with about 1,500 open reading frames (6, 14, 158, 197). Surprisingly, there are relatively few regulatory genes for an organism of its size. *H. pylori* is a panmictic species (187-188) exhibiting wide allelic diversity (87). The constant exchange of *H. pylori* DNA is aided by the naturally competent (150) nature of this organism. In addition, endogenous plasmids are not uncommon in *H. pylori* isolates with approximately 50% of isolates containing plasmids (167).

H. pylori has several virulence factors that enable the bacteria to effectively colonize the human host and enhance the pathogenesis of this organism. Upon entering the stomach, the bacteria must move through the gastric lumen, through the mucus layer to the underlying epithelial cells; thus, the flagella are essential for colonization (66). This organism also produces a mucinase to help breakdown the mucus layer in the stomach (181). Once through the mucus layer, approximately 20% of the bacteria adhere to the host cells (108) through adhesins like the sialic acid-binding adhesin (SabA) (133) and the Leb-binding adhesin (BabA) (28, 119) while the remaining bacteria continue to live within the mucus. As the stomach is an acidic environment, *H. pylori* must also have a mechanism for combating pH-related stress. This is primarily managed through the production of urease, an essential enzyme, which generates ammonia through the

Figure 1. Electron Micrograph of Wild-type H. pylori *strain G27.*

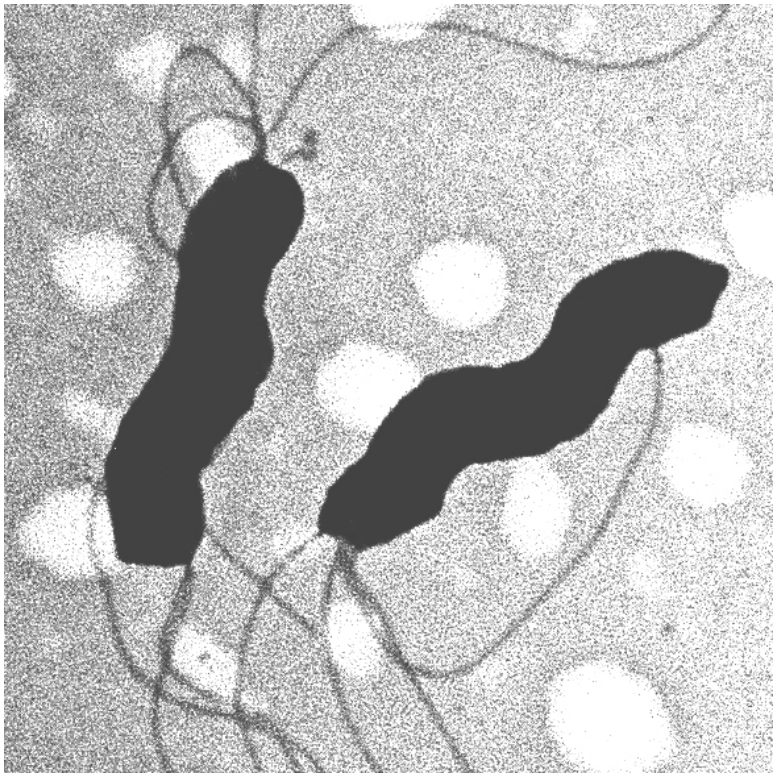


Photo used with permission of H. Gancz.

The characteristic spiral shape and polar flagella are distinctly visible.

hydrolysis of urea (64-65, 148). This ammonia helps neutralize the stomach acid and buffer the environment immediately surrounding the bacteria (64).

Virulence factors that directly target host cells and induce damage are produced once the bacteria have reached the site of infection. The most notable of these is the cytotoxin-associated gene A (CagA), which is encoded in some strains on a 40kb *cag* pathogenicity island (39). Also encoded on this pathogenicity island are the genes necessary to produce a type IV secretion system that is responsible for injecting CagA into the host cell (39, 156) and induces an interleukin-8 (IL-8) response in the host (10). Once inside the host cell, CagA is tyrosine phosphorylated by host cell kinases, binds to SHP-2, and modifies various host signaling cascades (109-111, 198). The injection of CagA also leads to morphological changes in the host cell, namely cell elongation referred to as the "hummingbird" phenotype (179). In addition to CagA, *H. pylori* also produces a toxin that is responsible for inducing vacuolization in host cells and is appropriately named the vacuolating cytotoxin (VacA) (46, 169, 193). In addition to its vacuolating properties, VacA has also been found to impact cell cycle regulation (136), alter the cytoskeleton (161), and induce autophagy (194) among other effects. In contrast to CagA and VacA, many of *H. pylori*'s virulence factors are poorly characterized. These include the neutrophil activating protein (NapA), which helps promote neutrophil recruitment and attachment to endothelial cells (77), the outer membrane protein, *homB* (122), the superoxide dismutase, *sodB* (180), and catalase (107). Despite its small genome, *H. pylori* is a highly effective pathogen.

Disease and epidemiology

When Marshall and Warren identified *H. pylori*, they were analyzing gastric biopsy specimens and noted that the bacteria were abundant in areas where inflammation was prominent (202) and that the presence of the bacteria was highly correlated with both gastric and duodenal ulcers (140). These initial observations, along with Marshall's infamous experiment where he consumed cultured *H. pylori* and documented his development of gastritis (137), led these two researchers to propose that this bacterium was the causative agent of acute gastritis, chronic gastritis, and peptic ulcers, a notion that would turn the medical world upside down (139). Up until the time of these studies, gastric maladies of these sorts were thought to be the result of stress or diet not a pathogen. In the present day, *H. pylori* is generally accepted to be the causative agent of gastritis and peptic ulcer disease, and is highly associated with two types of gastric cancer, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma (26, 165-166, 191). It is estimated that 50% of the world's population is infected with this organism (164), and while the majority of infections are asymptomatic, the number of infected individuals with clinical symptoms still presents a huge medical burden worldwide. *H. pylori* infections typically occur in childhood, and infection will remain life long unless the infected individual receives specific anti-*H. pylori* treatment. Due to the chronic tendancy of this infection, it is apparent why this pathogen is important. Due to its association with gastric cancer, *H. pylori* has been listed as a Class I carcinogen by the World Health Organization and is presently the only bacterium to be given that distinction (3).

Since it was first suggested to be the cause of the afore mentioned gastric diseases, determining how *H. pylori* causes disease has been a primary focus of the study of this organism. As described above, one of the original findings was that inflammation and the presence of *H. pylori* were closely linked. Gastritis, or inflammation of the gastric mucus membranes, is the most common disease outcome of infection with this organism with the majority of cases being subclinical. It is known that *H. pylori* elicits a strong IL-8 response in the host (47), which leads to the recruitment of neutrophils to the infection site. The NapA protein also helps to promote this neutrophilic response, and urease also evokes a proinflammatory response in the host (103). Other virulence factors like VacA and CagA damage host cells as described above and contribute to disease progression. Given the chronic nature of the infection, the immune system is constantly being stimulated, and over time an infected individual may progress to more severe disease development like peptic ulcers and gastric cancer. *H. pylori* infection is associated with 75% of gastric and 90% of duodenal ulcers, respectively (72). In considering the development of gastric cancer, being infected with a $CagA^+$ stain has been shown to be the most significant risk factor (27, 94). Given the number of infected individuals, is it perhaps not surprising that gastric cancer is the second leading cause of cancer-related deaths worldwide (151).

With the potential for severe disease outcome and half of the world's population infected, it would appear that the likelihood of any given person being infected is the same. However, this is not the case. Infection rates are highest in developing countries (2, 143), and declining rates among developed countries are associated with increased socioeconomic status. Still, worldwide infection usually occurs in childhood regardless

of socioeconomic status (64). Interestingly, evidence exists to suggest that overall *H. pylori* infection rates are slowly declining (15).

One critical piece of information that would greatly improve our understanding of the epidemiology of this organism is how *H. pylori* is transmitted. Since no reservoir of *H. pylori* has been found outside of the human (and non-human) primates it infects, the current view within the field is that person-to-person spread (oral-oral or fecal-oral) is the most likely mode of transmission (64, 147). Transmission is generally thought to occur primarily within families such that children born to at least one infected parent, especially if that parent is the mother, are far more likely to be colonized than those born to uninfected parents (126, 134, 176, 196). Despite our lack of understanding on how this organism is transmitted, *H. pylori* is clearly an efficient pathogen and one of medical importance.

Treatment and the future of H. pylori

The earliest report of effective treatment for *H. pylori* infection utilized bismuth and a systemic antibiotic (89). Today, treatment involves the use of a proton pump inhibitor and two antibiotics (clarithromycin and either metronidazole or amoxicillin) (135). Successful treatment results in the resolution of gastritis and ulceration. In fact, the first study to evaluate the effectiveness of *H. pylori* eradication in patients with duodenal ulcers was conducted in 1988 by Marshall; he found that among patients that cleared the infection, 92% of them also showed healing of ulcers with a relapse rate of only 21% within one year (138). In addition, antibiotic treatment has also been found to be effective in resolving *H. pylori* associated MALT lymphoma (183, 189). Due to the

risk of gastric cancer development in patients with *H. pylori* infections, it is the precedent to treat the infection to eliminate the risk of severe disease outcome. However, as with the treatment of most bacterial infections, antibiotic resistance is becoming a problem in the management of *H. pylori*-related diseases underscoring the need to develop new and better drug therapies and possibly a vaccine for this pathogen (121).

It is critical that effective therapies remain available for people with symptomatic *H. pylori* infections, but the question remains as to whether or not this organism should be eradicated from individuals who are not exhibiting clinical symptoms of disease. There is a growing body of evidence to suggest that *H. pylori* colonization may be beneficial for humans. For instance, it has been shown that the rate of *H. pylori* infection is inversely correlated to the rate of allergies and asthma among children in the United States, which is on the rise (42). This data fits in with the current "hygiene hypothesis" (186) that posits that as living environments have become more sanitary (germ-free), the incidence in allergic diseases has increased due to alterations in immune stimulation during childhood. Another example of the potential detriment to *H. pylori* eradication is the increasing rate of adenocarcinoma of the esophagus, which in the United States has been linked to the decrease in *H. pylori* infection (29, 67). The current thought behind this is best explained in a recent model of co-evolution of humans and *H. pylori* by Atherton and Blaser (9). This model proposes that we have not only evolved with the bacteria but that we have come to rely on them to help control gastric acid production. Therefore, if *H. pylori* is eliminated in individuals who have no overt disease symptoms, they will produce more stomach acid than normal thus increasing the risk of esophageal cancer (9). Another example of how *H. pylori* infection may provide an advantage to the

human host was reported in a recent study that examined the relationship between *H. pylori* infection and tuberculosis. This study showed that individuals who progressed to active tuberculosis disease during follow-up were far less likely to be infected with *H. pylori* than those who did not progress to active disease. Additionally, individuals with latent tuberculosis infections were more likely to be infected with *H. pylori* than individuals who do not have latent tuberculosis infections (168). In addition, after tuberculosis challenge, cynomolgus macaques that were naturally infected with *H. pylori* were less likely to develop active tuberculin disease than their non-*H. pylori* infected counterparts (168). The authors of this study suggest that chronic infection with *H. pylori* may enhance the body's ability to control *Mycobacterium tuberculosis* infection (168). Since these theories are considered to be controversial, only time and many more studies will reveal whether they are true.

In 2005, Warren and Marshall were awarded the Nobel Prize in Medicine for their discovery of *H. pylori* and their work in elucidating its role in the development of gastritis and peptic ulcer disease. So almost 30 years after the initial discovery of *H. pylori*, it can be argued that the accidental prolonged incubation of some biopsy plates was a very fortunate accident indeed.

The Ferric Uptake Regulator and Iron[1]

Background

In the struggle between host and pathogen, competition for resources is often a key point in determining who will be the ultimate winner. The goal of the pathogen is to

[1] Excerpts taken from the review article: Carpenter, B.M., J.M. Whitmire, and D.S. Merrell. This is Not Your Mother's Repressor: The Complex Role of Fur in Pathogenesis. 2009. Infect and Immun. 77: 2590-2601.

secure the necessary resources, often nutrients, from the host, while the goal of the host is to sequester the utilizable resources from the pathogen to help prevent infection. Among the key nutrients necessary to virtually all forms of life is iron. Iron plays an essential role in a diverse number of cellular processes. For instance, it serves as an enzymatic co-factor in metabolism and for electron transport. Thus, obtaining sufficient amounts of iron and maintaining iron stores is a critical function for both pathogen and host. However, having too much iron can be detrimental as excess iron can lead to the formation of hydroxyl radicals through Fenton chemistry, which in turn may lead to cellular and DNA damage.

Based on this yin-yang relationship, it is perhaps no surprise that the human host has several mechanisms for sequestering iron. In the body, iron is stored primarily in ferritin and hemosiderin while the majority of functional iron is found in hemoglobin (8). This being said there are several other iron storage molecules, like lactoferrin and transferrin, that sequester iron at the mucosal surfaces and within the circulatory system, respectively, and have been found to be iron sources for some pathogens (146). Global sequestration of free iron prevents possible oxidative damage as well as prevents its easy acquisition by pathogenic microbes. Additionally, in a further attempt to limit iron availability to pathogens during infection, the host decreases iron absorption from the gut, increases production of iron storage molecules, and shifts iron from the plasma into the storage molecules (204). Also, iron storage molecules are positioned in areas that are likely to be sites of infection. Thus, the host is immediately able to remove iron from those sites if a pathogen is detected (204). This process of removing free iron and other

nutrients from the body and containing them in various storage molecules is termed "nutritional immunity" (205).

Despite these well orchestrated defenses, bacterial pathogens have evolved mechanisms to breach the iron stores as well as to compete with the host for free iron. Proof of the pathogens success can be found by the strong connection between host iron overload and increased susceptibility to several bacterial infections. Indeed in a recent review of 67 years of medical literature, Khan, et al. found an increased association between infection with bacterial pathogens such as *Escherichia coli*, *Listeria monocytogenes*, and *Vibrio vulnificus* and hemochromatosis (125). In addition, iron overload in haemodialysis patients is associated with an increased number of bacterial infections as well as an increase in septicemic episodes (195). The importance of iron and infection in humans has also been validated for multiple pathogens using animal models. For instance, in a murine model of *V. vulnificus* infection, there is a drastic decrease in the LD_{50} from 10^6 to 1.1 bacterial cells in mice injected with extra iron (207). Also, *L. monocytogenes* exhibits increased growth *in vivo* and also displays a decreased LD_{50} in mice given additional iron (190). Finally, when murine infection models for both *Neisseria meningitidis* (114) and *Salmonella enterica* serovar Typhimurium (127) are given excess iron, infection is enhanced. While this is by no means an exhaustive list, it is clear that excess iron in the host helps to create a more hospitable environment for opportunistic pathogens. This is likely due to an increase in available free iron and potentially a decrease in anti-bacterial leukocyte activities (204).

In the midst of the mounting evidence for the connection between iron availability and increased susceptibility to bacterial infection, a mutant of *S. typhimurium* was

isolated that showed constitutive high level expression of the iron-enterochelin and ferrichrome iron uptake systems (71). This mutant was given the name *fur* for iron (Fe) uptake regulation (71) and today represents what we know as the ferric uptake regulator. The first *fur* mutants of *E. coli* were identified in 1981 and showed constitutive expression of *cir*, *fhuA*, and *fecA*, three iron uptake systems that are typically upregulated when available iron is low (102). Within one year, *E. coli fur* complementation studies showed that *fur* carried on a F' *lac* plasmid restored the wild-type phenotype in a strain bearing a chromosomal *fur* mutation (101). *E. coli fur* was successfully cloned in 1984 (100), and the gene sequence was derived shortly thereafter (177). Sequence and biochemical analyses went on to show that Fur is conserved across a wide range of bacterial species and is a small regulatory protein (15-17 kDa, approximately 150 amino acids (AAs) that functions as a dimer, is co-factored by Fe (II), and is usually autoregulatory.

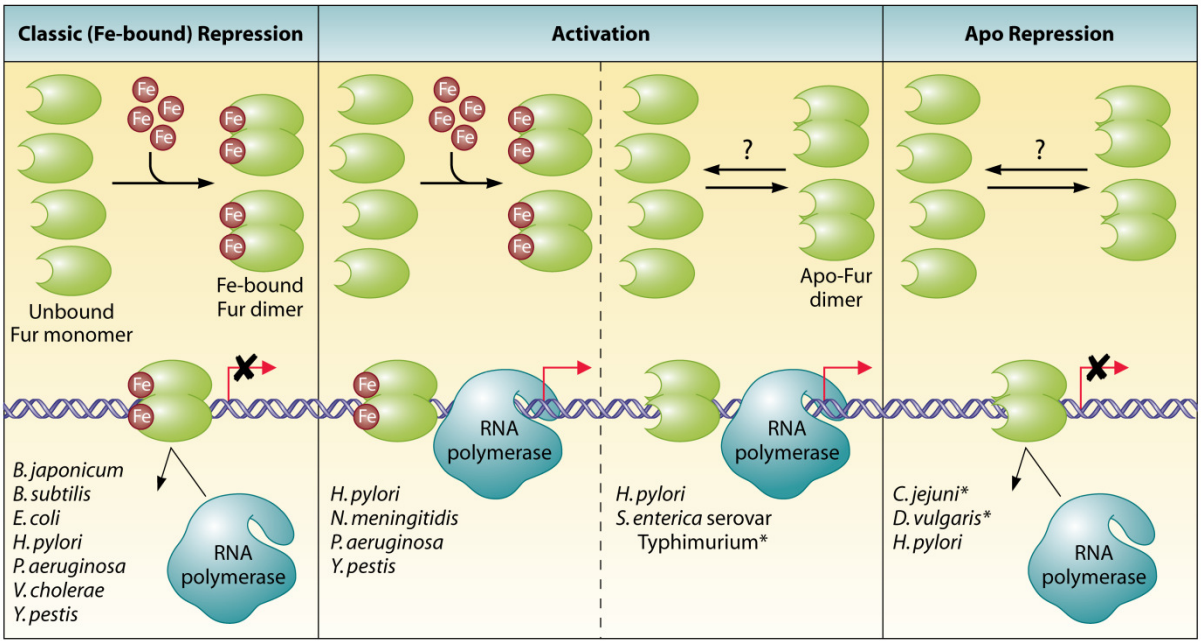
A greater understanding of the mechanism of Fur regulation came with the first description of a DNA binding consensus sequence for *E. coli* Fur (51). This 19-bp consensus sequence, GATAATGATAATCATTATC (51), would become the gold standard for comparison of Fur regulation across bacterial species and facilitated the understanding of exactly how Fur functions as a regulator: when iron is readily available in the bacterial cell, Fur binds iron, dimerizes, and the iron-bound Fur dimers bind to the consensus sequence in target promoters. Binding of Fur at the promoters prevents the binding of RNA polymerase; thus, transcription of the target gene is prevented. While Fur was first characterized as a transcriptional repressor under iron abundant conditions, it has subsequently been shown to function as an activator and even to repress certain

Figure 2. Basic Features of Fe-Fur repression, apo-*Fur repression, and Fur activation.*

Characteristic features of each type of Fur regulation are shown as they interact with a target DNA promoter. Classical iron-bound Fur repression is depicted in the Left Panel. As iron becomes increasingly available in the bacterial cell, the Fe (II) co-factor binds to *apo*-Fur monomers, and these now iron-bound monomers dimerize. The iron-bound Fur dimers repress transcription by binding to the Fur-box in their target promoters and block the binding of RNA polymerase. In the Center Panel, iron-bound Fur and *apo*-Fur activation is depicted. On the left side of this panel, iron-bound Fur dimers are formed under conditions of iron abundance, and these dimers bind to Fur-boxes in their respective target promoters and activate gene transcription. On the right side of the Center Panel, *apo*-Fur dimers form under low iron conditions. These *apo*-Fur dimers bind to Fur-boxes in their target promoters and activate gene transcription. *apo*-Fur repression is depicted in the Right Panel. Under iron deplete conditions, Fur is in its *apo* form, and *apo*-Fur binds to the Fur boxes of its target promoters. This binding blocks the binding of RNA polymerase; hence, transcription is repressed. For simplicity sake, *apo*-Fur repression and activation are depicted as being mediated through an *apo*-Fur dimer although it is not know if *apo*-Fur functions as a monomer or a dimer. Abbreviated lists of organisms that utilize each type of Fur regulation are listed in each panel.

* Indicates organisms where *apo*-Fur regulation has been suggested, but direct interaction has not yet been determined.

Figure 2. Basic Features of Fe-Fur repression, apo-*Fur repression, and Fur activation.*



Carpenter, B.M., J.M. Whitmire, and D.S. Merrell. This is Not Your Mother's Repressor: The Complex Role of Fur in Pathogenesis. 2009. Infect and Immun 77: 2590-2601.

genes in the absence of the iron co-factor, and these diverse types of Fur regulation will be discussed in detail further below (Fig. 2). While *fur* regulation continued to be studied in *E. coli* and in a wide variety of bacteria, it would take 25 years before what is arguably the next big breakthrough in the study of this regulator occurred – the crystallization of *Pseudomonas aeruginosa* Fur (170). Having the crystal structure of this important regulatory protein has enabled researchers to begin to make connections between what is known from genetic studies to the actual structure of the protein.

Fur, Iron homeostasis, and Bacterial Survival

Since iron is an essential nutrient for nearly all bacterial life but deadly in excess quantities, Fur's regulation of iron uptake and storage genes plays a significant role in the lives of the diverse number of bacteria that utilize it. As *E. coli* Fur is among the best studied, there have been numerous publications detailing Fur regulation of iron uptake systems in this model organism. These include the ferric citrate transport system (*fecABCDE*), the ferrichrome-iron receptor (*fhuA*), the colicin I receptor (*cir*) (93, 100, 102), the regulator of the *fecA-E* operon (*fecIR*) (7), the ferrienterochelin receptor (*fepA*), the *fecA-E* operon (*fecIR*) (7), the ferrienterochelin receptor (*fepA*), the ferric ion uptake gene (*fiu*) (100), the aerobactin (*iucA*) operon (11, 51, 73, 76), and the divergent operons of the ferrienterochelin receptor-ferric enterobactin esterase (*fepA-fes*) (75, 117). Additionally, recent macroarray analysis of the iron-dependent and Fur-dependent regulons in *E. coli* have confirmed the previously characterized Fur regulatory targets as well as identified several new Fur targets (142).

The large number of iron uptake genes that have been found to be controlled by Fur regulation is not restricted to Gram negative bacteria. Indeed, iron uptake is also

regulated by Fur in the model Gram positive organism, *Bacillus subtilis*. The best characterized of these systems include the catecholate siderophore, *dhb*, and a gene involved in ferri-hydroximate transport, *fhuD* (31). Interestingly, despite the fact that *in vivo* repression of *dhb* is only seen in the presence of iron, *in vitro* Fur binds to the *dhb* promoter even in the absence of its iron co-factor, though with slightly less affinity than when iron is present (30). Like with *E. coli*, global microarray analysis identified as many as 20 Fur-regulated operons, the majority of which are involved in iron acquisition (13).

Countless studies have gone on to show that as with *E. coli* and *B. subtilis*, Fur plays an essential role in iron acquisition systems and many other homeostatic processes for numerous bacterial pathogens. While too numerous to exhaustively discuss here, a few key examples that illustrate genes involved in siderophore production, genes involved in iron acquisition from heme, and iron storage will be expounded upon. A more extensive list of pathogens that utilize Fur and their Fur-regulated genes is summarized in Table 1. However, once again due to space limitations and the large volume of research on this important regulator, this table is by no means an exhaustive list.

Siderophores are iron-binding proteins secreted by bacteria to acquire iron from the environment. In *P. aeruginosa*, Fur regulates the production of the siderophores pyoverdin and pyochelin (171). In fact, pyoverdin has been found in the sputum of cystic fibrosis patients infected with *P. aeruginosa* (95), and isolates from patients produce both siderophores (96). A siderophore transport system (*sir*) as well as the ferrichrome uptake operon (*fhu*) are also regulated by Fur in *Staphylococcus aureus* (116, 210). Next, Fur

Table 1. Diverse Fur Regulated Genes from Model Organisms and Bacterial Pathogens[a]

Organism	Gene	Type of Fur Regulation	Reference
B. subtilis	*dhb*, catecholate siderophore	Fe-Fur repression	(30-31)
	fhuD, ferri-hydroximate transport	Fe-Fur repression	(31)
B. japonicum	*irr*, haem biosynthetic pathway regulator	Fe-Fur repression	(83, 99)
C. jejuni	Cj0859c, hypothetical	Suspected *apo*-Fur repression	(115)
	Cj1364, fumarate hydratase	Suspected *apo*-Fur repression	(115)
E. coli	*bfr*, bacterioferritin	Indirect Fur activation	(141)
	cfaB, CFA/I, fimbrial adhesin	Fe-Fur repression	(123)
	cir, colicin I receptor	Fe-Fur repression	(93, 100, 102)
	fecABCDE, ferric citrate transport	Fe-Fur repression	(7, 93, 100, 102)
	fecIR, regulator of *fecABCDE* operon	Fe-Fur repression	(7)
	fepA, ferrienterochelin receptor	Fe-Fur repression	(75, 100, 117)
	fes, ferric enterobactin esterase	Fe-Fur repression	(75, 117)
	fhuA, ferrichrome-iron receptor	Fe-Fur repression	(100, 102)
	fiu, ferric ion uptake	Fe-Fur repression	(100)
	fur, ferric uptake regulator	Fe-Fur repression	(50)
	hly, hemolysin	Fe-Fur repression	(82)
	iha, IrgA homolog adhesin	Fe-Fur repression	(174)
	iucA, aerobactin	Fe-Fur repression	(11, 51, 73, 76)
	stxA and *stxB*, Shiga toxins	Fe-Fur repression	(33)
	sodA, Mn-containing superoxide dismutase	Fe-Fur repression	(19, 43, 104, 153, 172, 192)
	sodB, Fe-containing superoxide dismutase	Indirect Fur activation	(62-63, 153)

H. ducreyi	*hgbA,* hemoglobin binding protein	Fe-Fur repression	(38)
H. pylori	*amiE*, aliphatic amidase	Fe-Fur repression	(36, 199)
	ceu, periplasmic iron binding protein	Fe-Fur repression	(200)
	exbB, biopolymer transport protein	Fe-Fur repression	(53)
	fecA, ferric citrate transport	Fe-Fur repression	(200)
	feoB, ferrous iron transport	Fe-Fur repression	(200)
	frpB, iron uptake system	Fe-Fur repression	(54, 58, 200)
	fur, ferric uptake regulator	Fe-Fur repression, *apo*-Fur activation	(56-57)
	nifS, Fe-S cluster synthesis protein	Fe-Fur activation	(5)
	pfr, prokaryotic ferritin	*apo*-Fur repression	(22, 36, 58)
	sodB, Fe-containing superoxide dismutase	*apo*-Fur repression	(35, 70)
	vacA, vacuolating cytotoxin	indirect Fur repression	(84)
L. monocytogenes	*fri*, ferritin-like protein	Fe-Fur repression	(79)
	fur, ferric uptake regulator	Fur regulation	(128, 152)
	svpA-srtB, iron uptake locus	Fe-Fur repression	(152)
M. smegmatis	*kat*, catalase-peroxidase	Fe-Fur repression	(173, 212)
M. tuberculosis	*kat*, catalase-peroxidase	Fe-Fur repression	(173, 212)
N. gonorrhoeae	*fbpA,* periplasmic binding protein	Fe-Fur repression	(59)
	fur, ferric uptake regulator	Fe-Fur regulation	(178)
	opaA, opaB, opaC, opaD, opaF, opaG, opaJ, opaK, opaE, opaH, and *opaI,* opacity proteins	Fe-Fur regulation	(178)
	sod, superoxide dismutase	Fur activation	(178)
	thpA and *thpB*, transferrin receptors	Fe-Fur repression	(4)
	tonB, receptor	Fe-Fur repression	(178)
N. meningitidis	*norB,* nitric oxide reductase	Fe-Fur activation	(55)
	nuoA, NADH dehydrogenase I chain A	Fe-Fur activation	(55)

	pan1, (now referred to as *aniA*) anaerobically induced outer membrane protein	Fe-Fur activation	(55)
P. aeruginosa	*bfr*, bacterioferritin	Fe-Fur activation	(206)
	fhuA, ferrichrome-iron receptor	Fe-Fur repression	(154)
	katB, catalase	Indirect Fur repression	(106)
	pchR, pyochelin siderophore	Fe-Fur repression	(154)
	pfeR, enterobactin receptor regulator	Fe-Fur repression	(155)
	pvdS, alternative sigma factor	Fe-Fur repression	(154)
	sdh, succinate dehydrogenase	Indirect Fur activation	(206)
	sodA, Mn-containing superoxide dismutase	Fe-Fur repression	(105-106)
	sodB, Fe-containing superoxide dismutase	Indirect Fur activation	(206)
	tonB, receptor	Fe-Fur repression	(155)
	toxA, exotoxin A	Indirect Fur repression	(154, 171)
S. aureus	*fhuCBD*, ferrichrome-iron receptor	Fe-Fur repression	(116, 210)
	kat, catalase-peroxidase	Fur activation[b]	(116)
	sirABC, siderophore transport system	Fe-Fur repression	(116, 210)
S. coelicolor	*catC*, catalase-peroxidase	Fe-Fur repression	(97)
	fur, ferric uptake regulator	Fe-Fur repression	(97)
S. enterica serovar Typhimurium	*hmp*, flavohemoglobin	Fe-Fur repression	(48)
	iro-28, iron regulated protein	*apo*-Fur activation	(81, 98)
	mntH, bacterial homolog of mammalian natural-resistance-associated macrophage protien 1	Fe-Fur repression	(68, 118, 124)
	rfrA and *rfrB*, sRNA	Fe-Fur repression	(68)
	sodB, Fe-containing superoxide dismutase	Indirect Fur activation	(68)
V. cholerae	*hly*, hemolysin	Fe-Fur repression	(184)

	irgA, outer membrane protein	Fe-Fur repression	(88, 132)
V. vulnificus	*fur*, ferric uptake regulator	*apo*-Fur activation	(129)
	hupA, heme utilization gene	Fe-Fur repression	(131)
	vuuA, vulnibactin receptor	Fe-Fur repression	(203)
Y. pestis	*bfr*, bacterioferritin	Fe-Fur repression	(85)
	fhuCDB, ferrichrome-iron receptor	Fe-Fur repression	(85)
	feoAB, ferrous iron transport	Fe-Fur repression	(85)
	fepB, ferrienterochelin receptor	Fe-Fur repression	(85)
	ftnA, iron storage protein	Fe-Fur activation	(85)
	iucA, aerobactin biosynthesis protein	Fe-Fur repression	(85)
	katA, catalase	*apo*-Fur activation	(85)
	napF, ferredoxin-type protein	Fe-Fur activation	(85)
	tonB, receptor	Fe-Fur repression	(85)

[a] Due to the large volume of research on Fur, this table does not represent an exhaustive list of Fur-regulated genes.
[b] Not determined whether Fur activation is mediated through Fe-Fur or *apo*-Fur.

regulates genes involved in the acquisition of iron from unique host sources, like heme and transferrin. *V. vulnificus* Fur regulates *hupA*, a heme utilization gene (131), while the causative agent of human chancroid, *Haemophilus ducreyi*, utilizes Fur to control expression of *hgbA*, which encodes a protein involved in hemoglobin binding (38). It is interesting to note that even though pathogenic *Neisseria* species produce transferrin receptors (*tbpA* and *tbpB*) to bind host iron sources, rather than produce siderophores to scavenge iron directly, the host may be attempting to limit iron availability and to decrease colonization by producing antibodies to these receptors during infection (4). Finally, Fur regulation of iron storage molecules is also important for bacterial pathogenesis to help ensure that once iron is acquired from the host, it is stored for use by the bacteria and contained to prevent the toxic effects of excess free iron. Fri, the only identified ferritin-like protein in *L. monocytogenes*, is Fur-regulated (79) as is a seven gene locus, *svpA-srtB* that is likely involved in iron uptake (152). Additionally, *H. pylori* Fur has been shown to regulate genes involved in iron acquisition and storage. The *pfr* gene, which encodes a prokaryotic ferritin molecule, is repressed by Fur in the absence of iron in what is termed *apo*-Fur regulation (22, 58) while iron uptake systems encoded by *frpB* (54, 58, 200), *fecA* (200), *ceuE* (200), *feoB* (200), and *exbB* (53) have all been found to be repressed by Fur in the presence of iron.

Not only does Fur play a role in iron acquisition in animal pathogens, but it is important for plant pathogens as well. In *Pseudomonas syringae*, Fur represses siderophore production (40), and in *Bradyrhizobium japonicum*, Fur regulates *irr*, the regulator of the heme biosynthetic pathway (83, 99). Even from this limited list of

pathogens, it is clear that Fur is critical for iron acquisition and storage in a wide variety of bacterial species.

Fur and the Link to Virulence

Not only is Fur involved in regulating iron homeostasis, it is also more directly involved in colonization and virulence. In *H. pylori*, *fur* mutants are less efficient at colonization in a murine model of infection (32) and are easily outcompeted by wild-type bacteria in *in vivo* competition assays in a Mongolian Gerbil model of infection (84). Thus, while it is not an essential gene in *H. pylori*, Fur certainly provides an advantage in establishing colonization. In addition, *fur* mutants in several pathogens exhibit decreased virulence in animal models. A murine skin abscess model of *S. aureus* infection shows that *fur* mutants are attenuated (116). Likewise *fur* mutants of *L. monocytogenes* (175) and *C. jejuni* (162) show reduced virulence in murine and chick models of infection, respectively, as well as *Edwardsiella tarda fur* mutants in fish (201). Even in plant pathogens like *P. syringae*, Fur mutants show decreased virulence (40).

In addition to a role in colonization, Fur also regulates numerous genes that are important for bacterial pathogenesis. For instance, in *P. aeruginosa* Fur has been shown to be involved in toxin production, biofilm formation, and quorum sensing. Fur is believed to be indirectly involved in *toxA* expression since it does not interact with the promoter of either *toxA* or its regulator, *regAB* (154, 171). Although the role Fur plays in biofilm formation in *P. aeruginosa* is not well characterized, a *fur* mutant formed more mature biofilms as compared to the wild-type under iron-limited conditions (16). Finally, Fur is indirectly involved in the regulation of quorum sensing in *P. aeruginosa* through

the regulation of two sRNAs, *prrF1* and *prrF2*, which in turn regulate degradation enzymes for the precursor molecule to *Pseudomonas* Quinolone Signal (157).

In *V. cholerae*, Fur negatively regulates hemolysin production (184) and an outer membrane virulence determinant, *irgA* (88, 132), while in *N. gonorrhoeae*, Fur directly interacts with the promoters of all 11 *opa* genes, which encode outer membrane proteins that aid in adherence to and invasion of host cells (178). In *N. meningitidis*, Fur is implicated in the regulation of several genes associated with virulence (92), and heat shock proteins are deregulated in a *fur* mutant of *N. meningitidis* independent of the iron-Fur regulon (52). In *E. coli*, the Shiga toxins (Stxs), StxA and StxB (33), and hemolysin (82) are repressed by Fur. Additionally, in uropathogenic and enterohemorrhagic strains of *E. coli*, Fur negatively regulates the IrgA homolog adhesin (*Iha*) (174) while in enterotoxigenic *E. coli*, the fimbrial adhesin, CFA/I, is repressed by Fur (123). Finally, the vacuolating cytotoxin (*vacA*) of *H. pylori* has been shown to be indirectly regulated by Fur (84). Thus, Fur plays a role in colonization and virulence in a diverse number of pathogens.

Fur and Low pH

In addition to its role in regulation of virulence factors, Fur is also important for regulation of processes that are necessary for survival *in vivo* and thus are linked to virulence. For instance, Fur is an important regulator of genes involved in the acid resistance response. Arguably, this is best exemplified in *S. typhimurium* where Fur is involved in the acid tolerance response (ATR). Specifically, a *S. typhimurium fur* mutant is unable to mount an effective ATR at pH 5.8 (80). Therefore, *fur* mutants are more

sensitive to acid (pH 3.3), and several ATR genes are not induced at pH 5.8 in the absence of *fur* (80). Intriguingly, Fur's role in acid resistance appears to be independent of iron and its role in iron acquisition (98) as iron availability does not affect ATR and "iron-blind" *fur* mutants still display an acid resistant phenotype (98). Further work has shown that Fur is primarily involved in helping *S. typhimurium* combat organic acid stress but plays only a minor role in inorganic acid stress (18). Fur in *H. pylori* has also been implicated in regulating genes involved in fighting acid stress (25, 84, 144); in fact, when exposed to low pH, the number of genes in the Fur regulon is significantly increased (84). These genes include, *gluP* encoding a predicted glucose/galactose transporter, *ruvC* encoding a predicted Holliday junction endodeoxyribonuclease, *fliP* encoding a flagellar biosynthetic protein, and *amiE* encoding the aliphatic amidase (84). AmiE helps counteract acid stress through the production of ammonia as a by-product of the hydrolysis of aliphatic amides (199). While Fur in these organisms is not solely responsible for acid resistance, it certainly plays a significant role in helping the bacteria adapt and adjust to acidic conditions that would be encountered within the host.

Fur and Oxidative Stress

Another survival mechanism in which Fur has been found to play a role in pathogenesis is in fighting oxidative stress via regulation of genes, like catalase and superoxide dismutase, that help to combat toxic oxygen products. Catalases and hydroperoxidases convert peroxides into water and oxygen, and superoxide dismutases convert superoxide radicals into oxygen and peroxide. For instance, in several organisms the catalase (*kat*) gene, which encodes the catalase enzyme, is regulated by Fur. Fur

represses *katG*, a combined catalase-peroxidase, in both *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, and this regulation is predicted to be universal in all *Mycobacterium* species (173, 212). Some bacterial species like *Y. pestis* (85), *S. aureus* (116), and *P. aeruginosa* (106) also utilize Fur to activate *kat* expression. In *E. coli, fur* mutants are more susceptible to UVA irradiation oxidative damage due to decreased production of the hydroperoxidases (HPI and HPII) (113). The neutrophil activating protein (*napA*), which helps protect *H. pylori* from oxidative damage, is suspected to be under the control of Fur (44, 145, 159). In addition, *S. typhimurium* Fur helps the bacteria counteract the effects of nitric oxide stress through the regulation of *hmp*, a flavohemoglobin (48). Also, of interest in this organism is Fur regulation of *mntH*, a gene that encodes a bacterial homolog of mammalian natural resistance associated macrophage protein 1 (118, 124). MntH is thought to help *S. typhimurium* fight hydrogen peroxide related injury upon entrance into macrophages (124).

Another oxidative survival gene that is commonly regulated by Fur is superoxide dismutase (*sod*). Sods are classified based on their metal co-factor: SodA, SodB, and SodC utilize, Mn (II), Fe (II), and Cu (II) or Zn (II), respectively, and the type of Sod or Sods varies with the bacterial species. In *N. gonorrhoeae*, Fur directly binds to the *sodB* promoter, which results in *sodB* activation (178). While in *E. coli, sodA* is directly repressed by Fur under iron abundant conditions (19, 43, 104, 153, 172, 192). In comparison, Fur regulation of the iron superoxide dismutase (*sodB*) in *E. coli* is indirectly activated by Fur (62-63, 153). As is seen here, Fur regulation of *sods* is exceptionally diverse, and to add to this mixture of direct or indirect activation and direct repression,

there is one more manner in which Fur has been shown to regulate *sod*. In *H. pylori*, *sodB* is directly repressed by Fur in the absence of iron, i.e. in its *apo* form (70).

It is evident that Fur is a global regulator that is involved in bacterial pathogenesis as well as many aspects of bacterial life (even some not described here e.g. Fur regulation of metabolic genes). As mentioned, classical Fur regulation involves the binding of iron-bound Fur dimers to the promoter region of target genes to occlude the RNA polymerase binding site; however, as also mentioned above, recent studies have shown instances where Fur functions as an activator or as a repressor in the absence of its iron co-factor. Presently, only one organism utilizes Fur in all of these different ways – *H. pylori*. While iron-bound Fur repression is well understood in this organism, *apo*-Fur repression and Fur activation comparatively remain in the proverbial "black box." In the remainder of this review we will highlight and compare the complexities of Fur regulation in this important human pathogen.

Helicobacter *and Iron-Associated Disease*

Interestingly, *H. pylori* infections are often associated with development of an iron deficiency anemia (IDA) that is usually unresponsive to iron replacement therapies (17). Two recent epidemiological studies highlighted this link by looking at adolescents and pregnant women: two groups of people who are at increased risk for IDA. During the adolescent years, an increased amount of iron is needed to support the rapid growth of the child, and similarly, during pregnancy, women need more iron due to increased blood volume and the iron needs of the developing fetus. In the first study, three adolescent children with IDA were unresponsive to iron supplementation (34). After the teens were

found to be infected with *H. pylori* and the infections were eradicated, the anemia resolved and iron levels returned to normal by three months post treatment (34). In the second study, a link between IDA in pregnancy and *H. pylori* infection was made. Out of 117 pregnant women, 27 of them had anemia; 18 were classified as suffering from IDA (149). All 27 of the anemic patients were shown to be *H. pylori* infected (149). The close association between *H. pylori* infection and IDA has prompted Cardamone, et al. to suggest that in cases of refractory IDA in teens, *H. pylori* infection should be considered as a diagnosis even in the absence of gastric symptoms (34).

While there is a strong epidemiological association between *H. pylori* and IDA, the mechanism by which the bacterium causes IDA is not known – is the bacterium directly removing iron from the host or is the severe inflammation associated with the infection the source of the iron loss? Several studies on *H. pylori* strains isolated from patients with IDA have attempted to explain the epidemiological association. Proteomic analysis of 15 strains (7 from IDA patients and 8 from non-IDA patients) revealed that IDA strains phylogenetically clustered together and separate from the non-IDA strains (163). Additionally, in a study of IDA strain isolates compared to non-IDA isolates, the strains from IDA patients showed increased uptake of both Fe (II) and Fe (III) (211) while the reason for this increased iron uptake is not known, certain polymorphisms in *feoB*, a ferrous iron transporter, have been shown to occur in IDA-derived strains of *H. pylori* (120). Even though the exact mechanism of how *H. pylori* and IDA are linked is not well understood, it is highly likely that Fur is playing some role in this process as it is the primary regulator of iron uptake and storage genes in this organism. The connection between IDA and Fur is likely to be mediated through Fur regulated genes and not

through Fur itself. Perhaps infected individuals who have IDA are infected with strains of *H. pylori* that have increased expression of iron uptake systems that are the result of altered Fur repression of these systems.

Iron-Bound Fur Repression

Fur was first identified in *H. pylori* in 1998 (24), is over 95% identical at the DNA and AA levels in *H. pylori* strains (23), and is 34% identical and 56% similar to *E. coli* Fur. Although Fur is nonessential in *H. pylori* (41) as discussed above, it is important for efficient colonization in both murine and gerbil animal models (32, 84). Based on the strong similarity to *E. coli*, it is likely no surprise that aspects of Fur regulation are similar between *H. pylori* and *E. coli*. As mentioned above, the best-described means of Fur regulation is classically hallmarked by iron-bound Fur dimers binding to specific regions in iron-regulated promoters called Fur-boxes (Fig. 2, Left Panel). Fur binding blocks the binding of RNA polymerase, thus preventing transcription of these target genes (73, 75). In *E. coli*, the Fur-box is a 19bp region, GATAATGATAATCATTATC, that is highly conserved in this organism (51). The *E. coli* Fur-box has also been reevaluated as 3 repeats of GATAAT with the second and third repeats separated by a single nucleotide and the last repeat inverted (74). Although the Fur-box of *E. coli* is used as the standard to which other Fur binding sequences are compared, it is not clearly conserved in all organisms that exhibit Fur regulation. For example, in *B. subtilis*, the Fur-box is a 15bp inverted repeat in a 7-1-7 configuration (12). Two of these motifs ($[7\text{-}1\text{-}7]_2$) may overlap to form the classic 19bp *E. coli* sequence (12). In *Y. pestis*, the Fur-box consists of two inverted repeats of

AATGATAAT separated by a single nucleotide (85). One common feature among Fur-boxes is the high number of A/T nucleotides relative to C/G nucleotides. Thus, in *H. pylori* it is perhaps no surprise that definition of a consensus Fur-box is somewhat hindered by the fact that it is a highly A/T rich organism (approximately 60%). Based on alignment of several Fur regulated genes, the consensus Fur-box in *H. pylori* is NNNNNAATAATNNTNANN (145). This consensus sequence is significantly different from *E. coli* and is certainly less conserved even among *H. pylori* Fur regulated genes than the *E. coli* sequence. While it is currently unclear, it may be that the requirement for Fur binding is less reliant on a recognition sequence and more related to the overall structural configuration of the target promoter sequence in *H. pylori.* This notion is further supported by the fact that *H. pylori* Fur is only partially able to complement an *E. coli fur* mutant (22) and that the *E. coli* Fur Titration Assay (FURTA-Ec) was not very successful at identifying Fur-regulated genes in *H. pylori* (20, 23-24), until the system was modified to allow *H. pylori* Fur expression (78).

Even though all of the specifics are not known, iron-bound Fur repression has been well documented in *H. pylori* and binding to several gene targets confirmed through DNase Footprinting analysis. Indeed, the predicted Fur regulon in *H. pylori* is quite extensive (49, 69, 145). The regulon includes iron uptake genes like *frpB* and *exbB* (53-54, 58) among others and *amiE* as well as other genes involved in functions like acid resistance (36, 199). Generally, iron-bound Fur regulated genes in *H. pylori* have one to three Fur binding sites within their promoters (53-54, 58). The sites with the highest affinity span the -10 and/or -35 promoter elements; the lesser affinity Fur binding sites are located further upstream from the primary Fur-box (53-54, 58). This high affinity

orientation supports the current hypothesis of Fur competing with RNA polymerase for binding to target promoters. Indeed, what we know about Fe-bound Fur regulation in *H. pylori* agrees with what is seen in many other organisms and is the most common mechanism of Fur regulation.

apo-*Fur Regulation*

Currently unique to *H. pylori* is the utilization of Fur as a repressor even in the absence of its Fe (II) co-factor. This phenomenon is termed *apo*-Fur regulation. It occurs under conditions of low iron availability and involves iron-free Fur binding to target promoters to prevent the binding of RNA polymerase. The *apo*-Fur regulon consists of an entirely different set of genes than the Fe-bound Fur regulon and is predicted to contain approximately 16 genes (69) though few genes have currently been definitively shown to be regulated in this manner. Expression of the iron storage molecule, Pfr, is regulated by *apo*-Fur (22, 36, 58); *pfr* expression is repressed under conditions of low iron but is constitutively expressed in a *fur* mutant (22). DNase I Footprinting analysis of the *pfr* promoter using iron-free Fur revealed that there were three regions of protection (58). Similar to iron-bound Fur repression, the region with the highest affinity for Fur covered the region to which RNA polymerase would bind (in this case both the -10 and -35 promoter elements, Fig. 2, Right Panel) (58). The other two regions were further upstream from the transcriptional start site (58). From a bacterial standpoint, repression of *pfr* under iron-limited conditions makes biological sense as producing a storage molecule when the molecule to be stored is not available would be a waste of energy and resources.

Another confirmed *apo*-Fur target is *sodB*. Binding of Fur to *sodB* in the absence of iron was shown via DNase I Footprinting analysis and Electrophoretic Mobility Shift Assays (EMSAs) (70). Unlike *pfr*, the *sodB* promoter has only one Fur binding region that spans the -10 and -35 promoter elements (70). Interestingly, comparison of the three *pfr* Fur-boxes and the *sodB* Fur-box shows very little sequence homology between them. Additionally, there is little homology with the known iron-bound *H. pylori* Fur-boxes and even less homology with the *E. coli* consensus Fur binding sequence (58, 70). Recent work from our group suggests that there are strain-specific nucleotide differences in the recognition sites in *apo*-Fur regulated promoters and that these differences may alter the affinity of *apo*-Fur for these promoters; a single nucleotide difference in the *sodB* Fur-box in strain G27 results in the loss of *sodB* regulation (37).

Even with the direct binding data provided by DNase I Footprinting and EMSA of the *pfr* and *sodB* promoters, the concept of *apo*-Fur regulation remains widely debated in the Fur field. The debate centers around whether or not Fur could actually be found unbound to iron *in vitro*. Is it possible to strip Fur of all of its iron co-factor in the laboratory? Some argue that the DNase Footprinting data are artificial because it is impossible to create *apo*-Fur *in vitro*; however, it is clear from mutational and transcriptional analyses that genes in the "*apo*-Fur" regulon are repressed in the absence of iron and constitutively expressed in a *fur* mutant, regardless of iron availability. One possibility is that the existence of Fur-regulated sRNAs, which control *apo* targets, could explain the *in vivo* data. sRNAs are a subclass of natural antisense transcripts that base-pair with complementary mRNA transcripts and thus can alter the stability of the mRNA or its ability to be translated (185). Up until very recently, there were no identified

sRNAs in *H. pylori*, but recently four have been identified (208-209). Two of the natural antisense transcripts in *H. pylori*, NAT-39 and NAT-67, were found to be complementary to *frpB* and *ceuE*, respectively (209). Both of these genes are members of the iron-bound Fur regulon (145). While it has been shown that NAT-39 and NAT-67 bind to their respective targets, it has not yet been determined what regulatory role this binding plays in gene expression and in iron homeostasis (209). The only other sRNAs identified in *H. pylori*, IG-443 and IG-524, are predicted to regulate the flagellar motor switch gene (*fliM*) and fumarase (*fumC*), respectively (208). Interestingly, IG-443 is encoded in the intergenic region between *fur* and *HP1033* (208). Given that the existence of sRNAs in *H. pylori* is a very recent discovery, the possibility of a sRNA that could regulate genes in the *apo*-Fur regulon cannot be ruled out, but to date there is no strong evidence of this being the case. Regardless, regulatory sRNA cannot account for the direct *in vitro* binding data demonstrated for *apo*-Fur and the *pfr* and *sodB* promoters.

Interestingly, there is some evidence that *apo*-Fur regulation may be found in other bacterial species. Microarray analysis of *C. jejuni* revealed that Cj1364, fumarate hydratase, and Cj0859c, a hypothetical protein, had reduced expression under iron replete conditions and had increased expression in the *fur* mutant (115). More recently, microarray analysis of the non-pathogen, *Desulfovibrio vulgaris* Hildenborough, predicted that there are nine genes that are repressed by iron-free Fur (21). More specific studies will need to be performed to determine whether *apo*-Fur regulation is actually occurring in these organisms.

Autoregulation of Fur

While some organisms have additional regulatory proteins to regulate Fur expression, like the catabolite-activator protein (CAP) in *E. coli* (50), RpoS in *V. vulnificus* (130), and NikR in *H. pylori* (53), autoregulation of Fur is the most conserved mechanism of *fur* regulation. Fur represses its own expression under iron-replete conditions. Biologically speaking, it makes sense to link the expression of Fur to the level of available iron given the dangers of iron toxicity. Fur can be thought of as a rheostat that senses the available iron and responds by regulating its own expression accordingly (56-57). It was determined early on that *E. coli* Fur was autoregulatory (50). Similarly, Fur from *E. tarda* (201) and *N. gonorrhoeae* (178) are autoregulatory, and Fur from *S. coelicolor* is predicted to be autoregulatory (97). In all of these instances, Fur autoregulation is the straightforward classical iron-bound Fur repression. However, in some organisms, Fur autoregulation appears to be more complex. For example, in *L. monocytogenes*, Northern Blot analysis reveals that *fur* is up-regulated under iron-limited conditions, yet *in vitro* DNase I Footprinting analysis shows that Fur is able to bind to and protect the Fur-box region of the *fur* promoter in the absence of the metal co-factor (128). The authors suggest that these results indicate that Fur binding is also dependent on an as yet unidentified factor (128). In contrast to this unidentified factor and iron-bound Fur autoregulation, *V. vulnificus* Fur has been shown to bind to and activate *fur* expression in the absence of iron (129).

Fur autoregulation in *H. pylori* may very well be the most complex Fur autoregulatory circuit known to date since it combines both the classical iron-bound Fur repression and the *apo*-Fur activation that is exhibited in *V. vulnificus*. Initial studies by

Delany, et al. revealed that there were three Fur binding regions in the *H. pylori fur* promoter. In order from highest to lowest affinity for Fur, operator I spans nucleotides -34 to -66, operator II spans nucleotides +19 to -13, and operator III spans nucleotides -87 to -104 (56). The first two operators are likely to be involved in repression of the *fur* promoter as they encompass both the -10 and -35 promoter elements, but the role of the third and farthest upstream operator was initially unclear (56). In their subsequent work, Delany, et al. showed that the third operator region was indeed important for Fur autoregulation and that it functions as a site for *apo*-Fur activation (similar to *V. vulnificus*). Additionally, operator I is involved in both iron-bound repression and *apo*-Fur activation of expression through binding Fur in its respective forms (57). Which form binds is driven by the prevalence of iron as both forms bind with equal affinity to this operator. The current model of *H. pylori* Fur autoregulation also suggests that if the concentration of Fur dips below a certain level, then Fur binding to operator I is lost, allowing this site to act as an UP element for RNA polymerase (57). Given that this organism utilizes Fur in both its iron-bound and *apo* forms, it is perhaps not surprising that Fur autoregulation in *H. pylori* is a complex mixture of iron-bound Fur repression and *apo*-Fur activation. Additionally, with few regulatory proteins relative to its genome size, *H. pylori* would likely have evolved to utilize every regulatory mechanism it has to ensure proper homeostasis.

Fur Activation

The complexity of *fur* autoregulation in *H. pylori* points to yet another regulatory function of Fur; Fur can act as a positive regulator. The first indication that Fur may be

acting as a positive regulator came from microarray analyses where a number of genes were suggested to be Fur-induced (49, 69). Another gene, *oorD*, a ferredoxin-like protein, is suspected of being activated by Fe-Fur as its expression is decreased in the absence of iron, and EMSA shows that Fe-bound Fur binds to its promoter (84).

Despite this circumstantial evidence, the process of Fur activation in *H. pylori* is currently poorly understood with the exception of *nifS*. NifS is a Fe-S cluster synthesis protein, which has been shown to be activated by iron-bound Fur (5). EMSA analysis shows Fur binding to the *nifS* promoter in the presence of the Fe (II) substitute, Mn, and *nifS* expression is increased in the presence of iron (5). Interestingly, the two predicted Fur-boxes for *nifS* are located far upstream of the transcriptional start site in the *nifS* promoter (5), similar to the *apo*-Fur activation site within the *fur* promoter. It appears from the examples of *fur*, *nifS,* and possibly *oorD* that both iron-bound and *apo*-Fur can act as transcriptional activators in addition to acting as repressors.

While there is clearly much to be learned about Fur activation in *H. pylori*, Fur activation in other organisms is better understood. In *N. meningitidis*, microarray analysis suggested that Fur activates multiple genes in the presence of iron (52, 92). Moreover direct iron-bound activation of the NMB1436-38 operon (91-92), *pan1*, *norB*, and *nuoA* promoters (55) has been shown. As with *H. pylori*, the Fur-boxes for the Fur-activated genes in *N. meningitidis* are also located further upstream in the promoters (55, 91). *S. typhimurium* utilizes both iron-bound and *apo*-Fur to activate a subset of genes although whether this is direct or indirect activation remains unclear (81). Additionally, the iron regulated protein, IRO-28, appears to be activated by *apo*-Fur under iron limited

conditions in *S. typhimurium* (81, 98). In *P. aeruginosa*, direct iron-bound Fur activation has been identified for the bacterioferritin gene, *bfrB* (206).

It is clear from these examples that Fur activation does not occur in the same manner as Fur repression (Fig. 2, Center Panel). For both iron-bound and *apo*-Fur repression, the Fur-boxes are located near the transcriptional start site and usually span at least one of the key promoter elements. Binding at this location blocks the binding of RNA polymerase. In contrast, the Fur-boxes for Fur activated genes are all located far upstream from the transcriptional start site; thus, binding of RNA polymerase is not hindered. Perhaps by binding further upstream within the promoter, Fur is able to change the overall structure of the DNA enabling better binding of the RNA polymerase to help facilitate transcription.

Goal and Specific Aims

The goal of the work depicted within this dissertation was to explore Fur regulation in *H. pylori*. In particular, I sought to better understand the structure function relationships necessary for regulation; thus, I conducted a structure function analysis of this protein to determine which amino acids are critical for iron-bound and for *apo* regulation by Fur. We hypothesized that four general groups of amino acids would be found in this study: 1.) those critical for iron-bound regulation, 2.) those critical for *apo* regulation, 3.) those critical for both forms of regulation, and 4.) those not critical for regulation. The specific aims of this dissertation were to generate site specific amino acid mutations in the *H. pylori* Fur protein and to analyze the affect of those mutations on Fur function. These two aims are described in the fourth chapter of this work. In addition to those specific aims, as described in Chapter Two, additional work was conducted to develop a new

plasmid-based genetic tool for use in the study of *H. pylori*. Fur and Fur-regulated genes served as the proof of concept for that particular study. Finally, as described in the third chapter of this dissertation, a study that looked at strain specific differences in the *apo*-Fur regulation of the *sodB* gene of *H. pylori* was conducted. Taken together, this work explores Fur regulation in *H. pylori* and broadens our understanding of how this important regulatory protein functions.

References

1. 1989. *Campylobacter pylori* becomes *Helicobacter pylori*. Lancet **2:**1019-20.

2. 1993. Epidemiology of, and risk factors for, *Helicobacter pylori* infection among 3194 asymptomatic subjects in 17 populations. The EUROGAST Study Group. Gut **34:**1672-6.

3. 1994. Infection with *Helicobacter pylori*. IARC Monogr Eval Carcinog Risks Hum **61:**177-240.

4. **Agarwal, S., C. A. King, E. K. Klein, D. E. Soper, P. A. Rice, L. M. Wetzler, and C. A. Genco.** 2005. The gonococcal Fur-regulated *tbpA* and *tbpB* genes are expressed during natural mucosal gonococcal infection. Infect Immun **73:**4281-7.

5. **Alamuri, P., N. Mehta, A. Burk, and R. J. Maier.** 2006. Regulation of the *Helicobacter pylori* Fe-S cluster synthesis protein NifS by iron, oxidative stress conditions, and Fur. J Bacteriol **188:**5325-30.

6. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-

sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.

7. **Angerer, A., and V. Braun.** 1998. Iron regulates transcription of the *Escherichia coli* ferric citrate transport genes directly and through the transcription initiation proteins. Arch Microbiol **169:**483-90.

8. **Aster, J. C.** 2005. Red blood cell and bleeding disorders, p. 619-659. *In* A. K. A. V. Kumar, and N. Fausto (ed.), Pathologic basis of disease, 7 ed. Elsevier Saunders, Philadelphia, PA.

9. **Atherton, J. C., and M. J. Blaser.** 2009. Coadaptation of *Helicobacter pylori* and humans: ancient history, modern implications. J Clin Invest **119:**2475-87.

10. **Backhed, F., E. Torstensson, D. Seguin, A. Richter-Dahlfors, and B. Rokbi.** 2003. *Helicobacter pylori* infection induces interleukin-8 receptor expression in the human gastric epithelium. Infect Immun **71:**3357-60.

11. **Bagg, A., and J. B. Neilands.** 1987. Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry **26:**5471-7.

12. **Baichoo, N., and J. D. Helmann.** 2002. Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. J Bacteriol **184:**5826-32.

13. **Baichoo, N., T. Wang, R. Ye, and J. D. Helmann.** 2002. Global analysis of the *Bacillus subtilis* Fur regulon and the iron starvation stimulon. Mol Microbiol **45:**1613-29.

14. **Baltrus, D. A., M. R. Amieva, A. Covacci, T. M. Lowe, D. S. Merrell, K. M. Ottemann, M. Stein, N. R. Salama, and K. Guillemin.** 2009. The complete genome sequence of *Helicobacter pylori* strain G27. J Bacteriol **191:**447-8.

15. **Banatvala, N., K. Mayo, F. Megraud, R. Jennings, J. J. Deeks, and R. A. Feldman.** 1993. The cohort effect and *Helicobacter pylori*. J Infect Dis **168:**219-21.

16. **Banin, E., M. L. Vasil, and E. P. Greenberg.** 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci U S A **102:**11076-81.

17. **Barabino, A.** 2002. *Helicobacter pylori*-related iron deficiency anemia: a review. Helicobacter **7:**71-5.

18. **Bearson, B. L., L. Wilson, and J. W. Foster.** 1998. A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. J Bacteriol **180:**2409-17.

19. **Beaumont, M. D., and H. M. Hassan.** 1993. Characterization of regulatory mutations causing anaerobic derepression of the *sodA* gene in *Escherichia coli* K12: cooperation between cis- and trans-acting regulatory loci. J Gen Microbiol **139:**2677-84.

20. **Beier, D., G. Spohn, R. Rappuoli, and V. Scarlato.** 1997. Identification and characterization of an operon of *Helicobacter pylori* that is involved in motility and stress adaptation. J Bacteriol **179:**4676-83.

21. **Bender, K. S., H. C. Yen, C. L. Hemme, Z. Yang, Z. He, Q. He, J. Zhou, K. H. Huang, E. J. Alm, T. C. Hazen, A. P. Arkin, and J. D. Wall.** 2007. Analysis of a ferric uptake regulator (Fur) mutant of *Desulfovibrio vulgaris* Hildenborough. Appl Environ Microbiol **73:**5389-400.

22. **Bereswill, S., S. Greiner, A. H. van Vliet, B. Waidner, F. Fassbinder, E. Schiltz, J. G. Kusters, and M. Kist.** 2000. Regulation of ferritin-mediated cytoplasmic iron storage by the ferric uptake regulator homolog (Fur) of *Helicobacter pylori*. J Bacteriol **182:**5948-53.

23. **Bereswill, S., F. Lichte, S. Greiner, B. Waidner, F. Fassbinder, and M. Kist.** 1999. The ferric uptake regulator (Fur) homologue of *Helicobacter pylori*: functional analysis of the coding gene and controlled production of the recombinant protein in *Escherichia coli*. Med Microbiol Immunol **188:**31-40.

24. **Bereswill, S., F. Lichte, T. Vey, F. Fassbinder, and M. Kist.** 1998. Cloning and characterization of the *fur* gene from *Helicobacter pylori*. FEMS Microbiol Lett **159:**193-200.

25. **Bijlsma, J. J., B. Waidner, A. H. Vliet, N. J. Hughes, S. Hag, S. Bereswill, D. J. Kelly, C. M. Vandenbroucke-Grauls, M. Kist, and J. G. Kusters.** 2002. The *Helicobacter pylori* homologue of the ferric uptake regulator is involved in acid resistance. Infect Immun **70:**606-11.

26. **Blaser, M. J.** 1998. *Helicobacter pylori* and gastric diseases. BMJ **316:**1507-10.

27. **Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M. Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura.** 1995. Infection with *Helicobacter pylori* strains possessing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res **55:**2111-5.

28. **Boren, T., P. Falk, K. A. Roth, G. Larson, and S. Normark.** 1993. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science **262:**1892-5.

29. **Brown, L. M., S. S. Devesa, and W. H. Chow.** 2008. Incidence of adenocarcinoma of the esophagus among white Americans by sex, stage, and age. J Natl Cancer Inst **100:**1184-7.

30. **Bsat, N., and J. D. Helmann.** 1999. Interaction of *Bacillus subtilis* Fur (ferric uptake repressor) with the *dhb* operator in vitro and in vivo. J Bacteriol **181:**4299-307.

31. **Bsat, N., A. Herbig, L. Casillas-Martinez, P. Setlow, and J. D. Helmann.** 1998. *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol Microbiol **29:**189-98.

32. **Bury-Mone, S., J. M. Thiberge, M. Contreras, A. Maitournam, A. Labigne, and H. De Reuse.** 2004. Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. Mol Microbiol **53:**623-38.

33. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the fur locus. J Bacteriol **169:**4759-64.

34. **Cardamone, M., G. Alex, M. D. Harari, W. P. Moss, and M. R. Oliver.** 2008. Severe iron-deficiency anaemia in adolescents: consider *Helicobacter pylori* infection. J Paediatr Child Health **44:**647-50.

35. **Carpenter, B. M., H. Gancz, R. P. Gonzalez-Nieves, A. L. West, J. M. Whitmire, S. L. Michel, and D. S. Merrell.** 2009. A single nucleotide change affects Fur-dependent regulation of *sodB* in *H. pylori*. PLoS One **4:**e5369.

36. **Carpenter, B. M., T. K. McDaniel, J. M. Whitmire, H. Gancz, S. Guidotti, S. Censini, and D. S. Merrell.** 2007. Expanding the *Helicobacter pylori* genetic toolbox: modification of an endogenous plasmid for use as a transcriptional reporter and complementation vector. Appl Environ Microbiol **73:**7506-14.

37. **Carpenter, B. M., J. M. Whitmire, and D. S. Merrell.** 2009. This is not your mother's repressor: the complex role of Fur in pathogenesis. Infect Immun **77:**2590-601.

38. **Carson, S. D., C. E. Thomas, and C. Elkins.** 1996. Cloning and sequencing of a *Haemophilus ducreyi fur* homolog. Gene **176:**125-9.

39. **Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci.** 1996. Cag, a pathogenicity island of *Helicobacter pylori,* encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A **93:**14648-53.

40. **Cha, J. Y., J. S. Lee, J. I. Oh, J. W. Choi, and H. S. Baik.** 2008. Functional analysis of the role of Fur in the virulence of *Pseudomonas syringae* pv. tabaci 11528: Fur controls expression of genes involved in quorum-sensing. Biochem Biophys Res Commun **366:**281-7.

41. **Chalker, A. F., H. W. Minehart, N. J. Hughes, K. K. Koretke, M. A. Lonetto, K. K. Brinkman, P. V. Warren, A. Lupas, M. J. Stanhope, J. R. Brown, and P. S. Hoffman.** 2001. Systematic identification of selective essential genes in *Helicobacter pylori* by genome prioritization and allelic replacement mutagenesis. J Bacteriol **183:**1259-68.

42. **Chen, Y., and M. J. Blaser.** 2008. *Helicobacter pylori* colonization is inversely associated with childhood asthma. J Infect Dis **198:**553-60.

43. **Compan, I., and D. Touati.** 1993. Interaction of six global transcription regulators in expression of manganese superoxide dismutase in *Escherichia coli* K-12. J Bacteriol **175:**1687-96.

44. **Cooksley, C., P. J. Jenks, A. Green, A. Cockayne, R. P. Logan, and K. R. Hardie.** 2003. NapA protects *Helicobacter pylori* from oxidative stress damage, and its production is influenced by the ferric uptake regulator. J Med Microbiol **52:**461-9.

45. **Covacci, A., J. L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli.** 1999. *Helicobacter pylori* virulence and genetic geography. Science **284:**1328-33.

46. **Cover, T. L., C. P. Dooley, and M. J. Blaser.** 1990. Characterization of and human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolizing cytotoxin activity. Infect Immun **58:**603-10.

47. **Crabtree, J. E., J. I. Wyatt, L. K. Trejdosiewicz, P. Peichl, P. H. Nichols, N. Ramsay, J. N. Primrose, and I. J. Lindley.** 1994. Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa. J Clin Pathol **47:**61-6.

48. **Crawford, M. J., and D. E. Goldberg.** 1998. Regulation of the *Salmonella typhimurium* flavohemoglobin gene. A new pathway for bacterial gene expression in response to nitric oxide. J Biol Chem **273:**34028-32.

49. **Danielli, A., D. Roncarati, I. Delany, V. Chiarini, R. Rappuoli, and V. Scarlato.** 2006. In vivo dissection of the *Helicobacter pylori* Fur regulatory circuit by genome-wide location analysis. J Bacteriol **188:**4654-62.

50. **De Lorenzo, V., M. Herrero, F. Giovannini, and J. B. Neilands.** 1988. Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *Escherichia coli*. Eur J Biochem **173:**537-46.

51. **de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (fur) repressor. J Bacteriol **169:**2624-30.

52. **Delany, I., R. Grifantini, E. Bartolini, R. Rappuoli, and V. Scarlato.** 2006. Effect of *Neisseria meningitidis* Fur mutations on global control of gene transcription. J Bacteriol **188:**2483-92.

53. **Delany, I., R. Ieva, A. Soragni, M. Hilleringmann, R. Rappuoli, and V. Scarlato.** 2005. In vitro analysis of protein-operator interactions of the NikR and Fur metal-responsive regulators of coregulated genes in *Helicobacter pylori*. J Bacteriol **187:**7703-15.

54. **Delany, I., A. B. Pacheco, G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. Iron-dependent transcription of the *frpB* gene of *Helicobacter pylori* is controlled by the Fur repressor protein. J Bacteriol **183:**4932-7.

55. **Delany, I., R. Rappuoli, and V. Scarlato.** 2004. Fur functions as an activator and as a repressor of putative virulence genes in *Neisseria meningitidis*. Mol Microbiol **52:**1081-90.

56. **Delany, I., G. Spohn, A. B. Pacheco, R. Ieva, C. Alaimo, R. Rappuoli, and V. Scarlato.** 2002. Autoregulation of *Helicobacter pylori* Fur revealed by functional analysis of the iron-binding site. Mol Microbiol **46:**1107-22.

57. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2003. An anti-repression Fur operator upstream of the promoter is required for iron-mediated transcriptional autoregulation in *Helicobacter pylori*. Mol Microbiol **50:**1329-38.

58. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. Mol Microbiol **42:**1297-309.

59. **Desai, P. J., A. Angerer, and C. A. Genco.** 1996. Analysis of Fur binding to operator sequences within the *Neisseria gonorrhoeae fbpA* promoter. J Bacteriol **178:**5020-3.

60. **Drazek, E. S., A. Dubois, and R. K. Holmes.** 1994. Characterization and presumptive identification of *Helicobacter pylori* isolates from rhesus monkeys. J Clin Microbiol **32:**1799-804.

61. **Dubois, A., N. Fiala, L. M. Heman-Ackah, E. S. Drazek, A. Tarnawski, W. N. Fishbein, G. I. Perez-Perez, and M. J. Blaser.** 1994. Natural gastric infection with *Helicobacter pylori* in monkeys: a model for spiral bacteria infection in humans. Gastroenterology **106:**1405-17.

62. **Dubrac, S., and D. Touati.** 2002. Fur-mediated transcriptional and post-transcriptional regulation of FeSOD expression in *Escherichia coli*. Microbiology **148:**147-56.

63. **Dubrac, S., and D. Touati.** 2000. Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. J Bacteriol **182:**3802-8.

64. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin Microbiol Rev **10:**720-41.

65. **Eaton, K. A., and S. Krakowka.** 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. Infect Immun **62:**3604-7.

66. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. J Med Microbiol **37:**123-7.

67. **el-Serag, H. B., and A. Sonnenberg.** 1998. Opposing time trends of peptic ulcer and reflux disease. Gut **43:**327-33.

68. **Ellermeier, J. R., and J. M. Slauch.** 2008. Fur regulates expression of the *Salmonella* pathogenicity island 1 type III secretion system through HilD. J Bacteriol **190:**476-86.

69. **Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. van Vliet, and G. Homuth.** 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. Microbiology **151:**533-46.

70. **Ernst, F. D., G. Homuth, J. Stoof, U. Mader, B. Waidner, E. J. Kuipers, M. Kist, J. G. Kusters, S. Bereswill, and A. H. van Vliet.** 2005. Iron-responsive

regulation of the *Helicobacter pylori* iron-cofactored superoxide dismutase SodB is mediated by Fur. J Bacteriol **187:**3687-92.

71. **Ernst, J. F., R. L. Bennett, and L. I. Rothfield.** 1978. Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of *Salmonella typhimurium*. J Bacteriol **135:**928-34.

72. **Ernst, P. B., and B. D. Gold.** 2000. The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. Annu Rev Microbiol **54:**615-40.

73. **Escolar, L., V. de Lorenzo, and J. Perez-Martin.** 1997. Metalloregulation in vitro of the aerobactin promoter of *Escherichia coli* by the Fur (ferric uptake regulation) protein. Mol Microbiol **26:**799-808.

74. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 1998. Binding of the Fur (ferric uptake regulator) repressor of *Escherichia coli* to arrays of the GATAAT sequence. J Mol Biol **283:**537-47.

75. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 1998. Coordinated repression in vitro of the divergent *fepA-fes* promoters of *Escherichia coli* by the iron uptake regulation (Fur) protein. J Bacteriol **180:**2579-82.

76. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 2000. Evidence of an unusually long operator for the Fur repressor in the aerobactin promoter of *Escherichia coli*. J Biol Chem **275:**24709-14.

77. **Evans, D. J., Jr., D. G. Evans, T. Takemura, H. Nakano, H. C. Lampert, D. Y. Graham, D. N. Granger, and P. R. Kvietys.** 1995. Characterization of a *Helicobacter pylori* neutrophil-activating protein. Infect Immun **63:**2213-20.

78. **Fassbinder, F., A. H. van Vliet, V. Gimmel, J. G. Kusters, M. Kist, and S. Bereswill.** 2000. Identification of iron-regulated genes of *Helicobacter pylori* by a modified Fur titration assay (FURTA-Hp). FEMS Microbiol Lett **184:**225-9.

79. **Fiorini, F., S. Stefanini, P. Valenti, E. Chiancone, and D. De Biase.** 2008. Transcription of the *Listeria monocytogenes fri* gene is growth-phase dependent and is repressed directly by Fur, the ferric uptake regulator. Gene **410:**113-21.

80. **Foster, J. W.** 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J Bacteriol **173:**6896-902.

81. **Foster, J. W., and H. K. Hall.** 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. J Bacteriol **174:**4317-23.

82. **Frechon, D., and E. Le Cam.** 1994. Fur (ferric uptake regulation) protein interaction with target DNA: comparison of gel retardation, footprinting and electron microscopy analyses. Biochem Biophys Res Commun **201:**346-55.

83. **Friedman, Y. E., and M. R. O'Brian.** 2003. A novel DNA-binding site for the ferric uptake regulator (Fur) protein from *Bradyrhizobium japonicum*. J Biol Chem **278:**38395-401.

84. **Gancz, H., S. Censini, and D. S. Merrell.** 2006. Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen *Helicobacter pylori*. Infect Immun **74:**602-14.

85. **Gao, H., D. Zhou, Y. Li, Z. Guo, Y. Han, Y. Song, J. Zhai, Z. Du, X. Wang, J. Lu, and R. Yang.** 2008. The iron-responsive Fur regulon in *Yersinia pestis*. J Bacteriol **190:**3063-75.

86. **Geis, G., S. Suerbaum, B. Forsthoff, H. Leying, and W. Opferkuch.** 1993. Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. J Med Microbiol **38:**371-7.

87. **Go, M. F., V. Kapur, D. Y. Graham, and J. M. Musser.** 1996. Population genetic analysis of *Helicobacter pylori* by multilocus enzyme electrophoresis:

extensive allelic diversity and recombinational population structure. J Bacteriol **178:**3934-8.

88. **Goldberg, M. B., S. A. Boyko, and S. B. Calderwood.** 1991. Positive transcriptional regulation of an iron-regulated virulence gene in *Vibrio cholerae*. Proc Natl Acad Sci U S A **88:**1125-9.

89. **Goodwin, C. S., J. A. Armstrong, and B. J. Marshall.** 1986. *Campylobacter pyloridis*, gastritis, and peptic ulceration. J Clin Pathol **39:**353-65.

90. **Goodwin, C. S., R. K. McCulloch, J. A. Armstrong, and S. H. Wee.** 1985. Unusual cellular fatty acids and distinctive ultrastructure in a new spiral bacterium (*Campylobacter pyloridis*) from the human gastric mucosa. J Med Microbiol **19:**257-67.

91. **Grifantini, R., E. Frigimelica, I. Delany, E. Bartolini, S. Giovinazzi, S. Balloni, S. Agarwal, G. Galli, C. Genco, and G. Grandi.** 2004. Characterization of a novel *Neisseria meningitidis* Fur and iron-regulated operon required for protection from oxidative stress: utility of DNA microarray in the assignment of the biological role of hypothetical genes. Mol Microbiol **54:**962-79.

92. **Grifantini, R., S. Sebastian, E. Frigimelica, M. Draghi, E. Bartolini, A. Muzzi, R. Rappuoli, G. Grandi, and C. A. Genco.** 2003. Identification of iron-

activated and -repressed Fur-dependent genes by transcriptome analysis of *Neisseria meningitidis* group B. Proc Natl Acad Sci U S A **100:**9542-7.

93. **Griggs, D. W., and J. Konisky.** 1989. Mechanism for iron-regulated transcription of the *Escherichia coli cir* gene: metal-dependent binding of Fur protein to the promoters. J Bacteriol **171:**1048-54.

94. **Gwack, J., A. Shin, C. S. Kim, K. P. Ko, Y. Kim, J. K. Jun, J. Bae, S. K. Park, Y. C. Hong, D. Kang, S. H. Chang, H. R. Shin, and K. Y. Yoo.** 2006. CagA-producing *Helicobacter pylori* and increased risk of gastric cancer: a nested case-control study in Korea. Br J Cancer **95:**639-41.

95. **Haas, B., J. Kraut, J. Marks, S. C. Zanker, and D. Castignetti.** 1991. Siderophore presence in sputa of cystic fibrosis patients. Infect Immun **59:**3997-4000.

96. **Haas, B., E. Murphy, and D. Castignetti.** 1991. Siderophore synthesis by mucoid *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients. Can J Microbiol **37:**654-7.

97. **Hahn, J. S., S. Y. Oh, and J. H. Roe.** 2000. Regulation of the *furA* and *catC* operon, encoding a ferric uptake regulator homologue and catalase-peroxidase, respectively, in *Streptomyces coelicolor* A3(2). J Bacteriol **182:**3767-74.

98. **Hall, H. K., and J. W. Foster.** 1996. The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. J Bacteriol **178:**5683-91.

99. **Hamza, I., Z. Qi, N. D. King, and M. R. O'Brian.** 2000. Fur-independent regulation of iron metabolism by Irr in *Bradyrhizobium japonicum*. Microbiology **146 (Pt 3):**669-76.

100. **Hantke, K.** 1984. Cloning of the repressor protein gene of iron-regulated systems in *Escherichia coli* K12. Mol Gen Genet **197:**337-41.

101. **Hantke, K.** 1982. Negative control of iron uptake systems in *Escherichia coli*. FEMS Microbiology Letters **15:**83-86.

102. **Hantke, K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol Gen Genet **182:**288-92.

103. **Harris, P. R., H. L. Mobley, G. I. Perez-Perez, M. J. Blaser, and P. D. Smith.** 1996. *Helicobacter pylori* urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology **111:**419-25.

104. **Hassan, H. M., and H. C. Sun.** 1992. Regulatory roles of Fnr, Fur, and Arc in expression of manganese-containing superoxide dismutase in *Escherichia coli*. Proc Natl Acad Sci U S A **89:**3217-21.

105. **Hassett, D. J., M. L. Howell, U. A. Ochsner, M. L. Vasil, Z. Johnson, and G. E. Dean.** 1997. An operon containing *fumC* and *sodA* encoding fumarase C and manganese superoxide dismutase is controlled by the ferric uptake regulator in *Pseudomonas aeruginosa*: fur mutants produce elevated alginate levels. J Bacteriol **179:**1452-9.

106. **Hassett, D. J., P. A. Sokol, M. L. Howell, J. F. Ma, H. T. Schweizer, U. Ochsner, and M. L. Vasil.** 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. J Bacteriol **178:**3996-4003.

107. **Hazell, S. L., D. J. Evans, Jr., and D. Y. Graham.** 1991. *Helicobacter pylori* catalase. J Gen Microbiol **137:**57-61.

108. **Hessey, S. J., J. Spencer, J. I. Wyatt, G. Sobala, B. J. Rathbone, A. T. Axon, and M. F. Dixon.** 1990. Bacterial adhesion and disease activity in *Helicobacter* associated chronic gastritis. Gut **31:**134-8.

109. **Higashi, H., A. Nakaya, R. Tsutsumi, K. Yokoyama, Y. Fujii, S. Ishikawa, M. Higuchi, A. Takahashi, Y. Kurashima, Y. Teishikata, S. Tanaka, T. Azuma, and M. Hatakeyama.** 2004. *Helicobacter pylori* CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. J Biol Chem **279:**17205-16.

110. **Higashi, H., R. Tsutsumi, A. Fujita, S. Yamazaki, M. Asaka, T. Azuma, and M. Hatakeyama.** 2002. Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proc Natl Acad Sci U S A **99:**14428-33.

111. **Higashi, H., R. Tsutsumi, S. Muto, T. Sugiyama, T. Azuma, M. Asaka, and M. Hatakeyama.** 2002. SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. Science **295:**683-6.

112. **Ho, S. A., J. A. Hoyle, F. A. Lewis, A. D. Secker, D. Cross, N. P. Mapstone, M. F. Dixon, J. I. Wyatt, D. S. Tompkins, G. R. Taylor, and et al.** 1991. Direct polymerase chain reaction test for detection of *Helicobacter pylori* in humans and animals. J Clin Microbiol **29:**2543-9.

113. **Hoerter, J. D., A. A. Arnold, C. S. Ward, M. Sauer, S. Johnson, T. Fleming, and A. Eisenstark.** 2005. Reduced hydroperoxidase (HPI and HPII) activity in

the Δ*fur* mutant contributes to increased sensitivity to UVA radiation in *Escherichia coli*. J Photochem Photobiol B **79:**151-7.

114. **Holbein, B. E., K. W. Jericho, and G. C. Likes.** 1979. *Neisseria meningitidis* infection in mice: influence of iron, variations in virulence among strains, and pathology. Infect Immun **24:**545-51.

115. **Holmes, K., F. Mulholland, B. M. Pearson, C. Pin, J. McNicholl-Kennedy, J. M. Ketley, and J. M. Wells.** 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology **151:**243-57.

116. **Horsburgh, M. J., E. Ingham, and S. J. Foster.** 2001. In *Staphylococcus aureus*, Fur is an interactive regulator with PerR, contributes to virulence, and Is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. J Bacteriol **183:**468-75.

117. **Hunt, M. D., G. S. Pettis, and M. A. McIntosh.** 1994. Promoter and operator determinants for *fur*-mediated iron regulation in the bidirectional *fepA-fes* control region of the *Escherichia coli* enterobactin gene system. J Bacteriol **176:**3944-55.

118. **Ikeda, J. S., A. Janakiraman, D. G. Kehres, M. E. Maguire, and J. M. Slauch.** 2005. Transcriptional regulation of *sitABCD* of *Salmonella enterica* serovar Typhimurium by MntR and Fur. J Bacteriol **187:**912-22.

119. **Ilver, D., A. Arnqvist, J. Ogren, I. M. Frick, D. Kersulyte, E. T. Incecik, D. E. Berg, A. Covacci, L. Engstrand, and T. Boren.** 1998. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science **279:**373-7.

120. **Jeon, B. H., Y. J. Oh, N. G. Lee, and Y. H. Choe.** 2004. Polymorphism of the *Helicobacter pylori feoB* gene in Korea: a possible relation with iron-deficiency anemia? Helicobacter **9:**330-4.

121. **Jones, K. R., Jeong-Heon Cha, and D. Scotty Merrell.** 2008. Who's Winning the War? Molecular Mechanisms of Antibiotic Resistance in *Helicobacter pylori*. Current Drug Therapy **3:**190.

122. **Jung, S. W., M. Sugimoto, D. Y. Graham, and Y. Yamaoka.** 2009. *homB* status of *Helicobacter pylori* as a novel marker to distinguish gastric cancer from duodenal ulcer. J Clin Microbiol **47:**3241-5.

123. **Karjalainen, T. K., D. G. Evans, D. J. Evans, Jr., D. Y. Graham, and C. H. Lee.** 1991. Iron represses the expression of CFA/I fimbriae of enterotoxigenic *E. coli*. Microb Pathog **11:**317-23.

124. **Kehres, D. G., A. Janakiraman, J. M. Slauch, and M. E. Maguire.** 2002. Regulation of *Salmonella enterica* serovar Typhimurium *mntH* transcription by H(2)O(2), Fe(2+), and Mn(2+). J Bacteriol **184:**3151-8.

125. **Khan, F. A., M. A. Fisher, and R. A. Khakoo.** 2007. Association of hemochromatosis with infectious diseases: expanding spectrum. Int J Infect Dis **11:**482-7.

126. **Kivi, M., A. L. Johansson, M. Reilly, and Y. Tindberg.** 2005. *Helicobacter pylori* status in family members as risk factors for infection in children. Epidemiol Infect **133:**645-52.

127. **Kochan, I., J. Wasynczuk, and M. A. McCabe.** 1978. Effects of injected iron and siderophores on infections in normal and immune mice. Infect Immun **22:**560-7.

128. **Ledala, N., S. L. Pearson, B. J. Wilkinson, and R. K. Jayaswal.** 2007. Molecular characterization of the Fur protein of *Listeria monocytogenes*. Microbiology **153:**1103-11.

129. **Lee, H. J., S. H. Bang, K. H. Lee, and S. J. Park.** 2007. Positive regulation of *fur* gene expression via direct interaction of Fur in a pathogenic bacterium, *Vibrio vulnificus*. J Bacteriol **189:**2629-36.

130. **Lee, H. J., K. J. Park, A. Y. Lee, S. G. Park, B. C. Park, K. H. Lee, and S. J. Park.** 2003. Regulation of *fur* expression by RpoS and Fur in *Vibrio vulnificus*. J Bacteriol **185:**5891-6.

131. **Litwin, C. M., and B. L. Byrne.** 1998. Cloning and characterization of an outer membrane protein of *Vibrio vulnificus* required for heme utilization: regulation of expression and determination of the gene sequence. Infect Immun **66:**3134-41.

132. **Litwin, C. M., and S. B. Calderwood.** 1994. Analysis of the complexity of gene regulation by Fur in *Vibrio cholerae*. J Bacteriol **176:**240-8.

133. **Mahdavi, J., B. Sonden, M. Hurtig, F. O. Olfat, L. Forsberg, N. Roche, J. Angstrom, T. Larsson, S. Teneberg, K. A. Karlsson, S. Altraja, T. Wadstrom, D. Kersulyte, D. E. Berg, A. Dubois, C. Petersson, K. E. Magnusson, T. Norberg, F. Lindh, B. B. Lundskog, A. Arnqvist, L. Hammarstrom, and T. Boren.** 2002. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science **297:**573-8.

134. **Malaty, H. M.** 2007. Epidemiology of *Helicobacter pylori* infection. Best Pract Res Clin Gastroenterol **21:**205-14.

135. **Malfertheiner, P., F. Megraud, C. O'Morain, F. Bazzoli, E. El-Omar, D. Graham, R. Hunt, T. Rokkas, N. Vakil, and E. J. Kuipers.** 2007. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. Gut **56:**772-81.

136. **Manente, L., A. Perna, E. Buommino, L. Altucci, A. Lucariello, G. Citro, A. Baldi, G. Iaquinto, M. A. Tufano, and A. De Luca.** 2008. The *Helicobacter pylori*'s protein VacA has direct effects on the regulation of cell cycle and apoptosis in gastric epithelial cells. J Cell Physiol **214:**582-7.

137. **Marshall, B. J., J. A. Armstrong, D. B. McGechie, and R. J. Glancy.** 1985. Attempt to fulfil Koch's postulates for pyloric Campylobacter. Med J Aust **142:**436-9.

138. **Marshall, B. J., C. S. Goodwin, J. R. Warren, R. Murray, E. D. Blincow, S. J. Blackbourn, M. Phillips, T. E. Waters, and C. R. Sanderson.** 1988. Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori*. Lancet **2:**1437-42.

139. **Marshall, B. J., D. B. McGechie, P. A. Rogers, and R. J. Glancy.** 1985. Pyloric Campylobacter infection and gastroduodenal disease. Med J Aust **142:**439-44.

140. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **1:**1311-5.

141. **Masse, E., and S. Gottesman.** 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc Natl Acad Sci U S A **99:**4620-5.

142. **McHugh, J. P., F. Rodriguez-Quinones, H. Abdul-Tehrani, D. A. Svistunenko, R. K. Poole, C. E. Cooper, and S. C. Andrews.** 2003. Global iron-dependent gene regulation in *Escherichia coli.* A new mechanism for iron homeostasis. J Biol Chem **278:**29478-86.

143. **Megraud, F., M. P. Brassens-Rabbe, F. Denis, A. Belbouri, and D. Q. Hoa.** 1989. Seroepidemiology of *Campylobacter pylori* infection in various populations. J Clin Microbiol **27:**1870-3.

144. **Merrell, D. S., M. L. Goodrich, G. Otto, L. S. Tompkins, and S. Falkow.** 2003. pH-regulated gene expression of the gastric pathogen *Helicobacter pylori*. Infect Immun **71:**3529-39.

145. **Merrell, D. S., L. J. Thompson, C. C. Kim, H. Mitchell, L. S. Tompkins, A. Lee, and S. Falkow.** 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. Infect Immun **71:**6510-25.

146. **Miethke, M., and M. A. Marahiel.** 2007. Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev **71:**413-51.

147. **Mitchell, H. M.** 2001. Epidemiology of Infection, p. 7-18. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* Physiology and Genetics. ASM Press, Washington, DC.

148. **Mobley, H. L., M. J. Cortesia, L. E. Rosenthal, and B. D. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. J Clin Microbiol **26:**831-6.

149. **Mulayim, B., N. Y. Celik, and F. F. Yanik.** 2008. *Helicobacter pylori* infection detected by 14C-urea breath test is associated with iron deficiency anemia in pregnant women. J Obstet Gynaecol Res **34:**980-5.

150. **Nedenskov-Sorensen, P., G. Bukholm, and K. Bovre.** 1990. Natural competence for genetic transformation in *Campylobacter pylori*. J Infect Dis **161:**365-6.

151. **Neugut, A. I., M. Hayek, and G. Howe.** 1996. Epidemiology of gastric cancer. Semin Oncol **23:**281-91.

152. **Newton, S. M., P. E. Klebba, C. Raynaud, Y. Shao, X. Jiang, I. Dubail, C. Archer, C. Frehel, and A. Charbit.** 2005. The *svpA-srtB* locus of *Listeria monocytogenes*: Fur-mediated iron regulation and effect on virulence. Mol Microbiol **55:**927-40.

153. **Niederhoffer, E. C., C. M. Naranjo, K. L. Bradley, and J. A. Fee.** 1990. Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. J Bacteriol **172:**1930-8.

154. **Ochsner, U. A., A. I. Vasil, and M. L. Vasil.** 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. J Bacteriol **177:**7194-201.

155. **Ochsner, U. A., and M. L. Vasil.** 1996. Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc Natl Acad Sci U S A **93:**4409-14.

156. **Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas.** 2000. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. Science **287:**1497-500.

157. **Oglesby, A. G., J. M. Farrow, 3rd, J. H. Lee, A. P. Tomaras, E. P. Greenberg, E. C. Pesci, and M. L. Vasil.** 2008. The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. J Biol Chem **283:**15558-67.

158. **Oh, J. D., H. Kling-Backhed, M. Giannakis, J. Xu, R. S. Fulton, L. A. Fulton, H. S. Cordum, C. Wang, G. Elliott, J. Edwards, E. R. Mardis, L. G. Engstrand, and J. I. Gordon.** 2006. The complete genome sequence of a chronic atrophic gastritis *Helicobacter pylori* strain: evolution during disease progression. Proc Natl Acad Sci U S A **103:**9999-10004.

159. **Olczak, A. A., G. Wang, and R. J. Maier.** 2005. Up-expression of NapA and other oxidative stress proteins is a compensatory response to loss of major *Helicobacter pylori* stress resistance factors. Free Radic Res **39:**1173-82.

160. **Owen, R. J.** 1998. *Helicobacter*--species classification and identification. Br Med Bull **54:**17-30.

161. **Pai, R., T. L. Cover, and A. S. Tarnawski.** 1999. *Helicobacter pylori* vacuolating cytotoxin (VacA) disorganizes the cytoskeletal architecture of gastric epithelial cells. Biochem Biophys Res Commun **262:**245-50.

162. **Palyada, K., D. Threadgill, and A. Stintzi.** 2004. Iron acquisition and regulation in *Campylobacter jejuni*. J Bacteriol **186:**4714-29.

163. **Park, S. A., H. W. Lee, M. H. Hong, Y. W. Choi, Y. H. Choe, B. Y. Ahn, Y. J. Cho, D. S. Kim, and N. G. Lee.** 2006. Comparative proteomic analysis of *Helicobacter pylori* strains associated with iron deficiency anemia. Proteomics **6:**1319-28.

164. **Parsonnet, J.** 1995. The incidence of *Helicobacter pylori* infection. Aliment Pharmacol Ther **9 Suppl 2:**45-51.

165. **Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med **325:**1127-31.

166. **Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman.** 1994. *Helicobacter pylori* infection and gastric lymphoma. N Engl J Med **330:**1267-71.

167. **Penfold, S. S., A. J. Lastovica, and B. G. Elisha.** 1988. Demonstration of plasmids in *Campylobacter pylori*. J Infect Dis **157:**850-1.

168. **Perry, S., B. C. de Jong, J. V. Solnick, M. de la Luz Sanchez, S. Yang, P. L. Lin, L. M. Hansen, N. Talat, P. C. Hill, R. Hussain, R. A. Adegbola, J. Flynn,**

D. Canfield, and J. Parsonnet. 2010. Infection with *Helicobacter pylori* is associated with protection against tuberculosis. PLoS One **5:**e8804.

169. **Phadnis, S. H., D. Ilver, L. Janzon, S. Normark, and T. U. Westblom.** 1994. Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. Infect Immun **62:**1557-65.

170. **Pohl, E., J. C. Haller, A. Mijovilovich, W. Meyer-Klaucke, E. Garman, and M. L. Vasil.** 2003. Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. Mol Microbiol **47:**903-15.

171. **Prince, R. W., C. D. Cox, and M. L. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J Bacteriol **175:**2589-98.

172. **Privalle, C. T., and I. Fridovich.** 1993. Iron specificity of the Fur-dependent regulation of the biosynthesis of the manganese-containing superoxide dismutase in *Escherichia coli*. J Biol Chem **268:**5178-81.

173. **Pym, A. S., P. Domenech, N. Honore, J. Song, V. Deretic, and S. T. Cole.** 2001. Regulation of catalase-peroxidase (KatG) expression, isoniazid sensitivity and virulence by *furA* of *Mycobacterium tuberculosis*. Mol Microbiol **40:**879-89.

174. **Rashid, R. A., P. I. Tarr, and S. L. Moseley.** 2006. Expression of the *Escherichia coli* IrgA homolog adhesin is regulated by the ferric uptake regulation protein. Microb Pathog **41:**207-17.

175. **Rea, R. B., C. G. Gahan, and C. Hill.** 2004. Disruption of putative regulatory loci in *Listeria monocytogenes* demonstrates a significant role for Fur and PerR in virulence. Infect Immun **72:**717-27.

176. **Rothenbacher, D., M. Winkler, T. Gonser, G. Adler, and H. Brenner.** 2002. Role of infected parents in transmission of *Helicobacter pylori* to their children. Pediatr Infect Dis J **21:**674-9.

177. **Schaffer, S., K. Hantke, and V. Braun.** 1985. Nucleotide sequence of the iron regulatory gene *fur*. Mol Gen Genet **200:**110-3.

178. **Sebastian, S., S. Agarwal, J. R. Murphy, and C. A. Genco.** 2002. The gonococcal Fur regulon: identification of additional genes involved in major catabolic, recombination, and secretory pathways. J Bacteriol **184:**3965-74.

179. **Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins.** 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. Proc Natl Acad Sci U S A **96:**14559-64.

180. **Seyler, R. W., Jr., J. W. Olson, and R. J. Maier.** 2001. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. Infect Immun **69:**4034-40.

181. **Smith, A. W., B. Chahal, and G. L. French.** 1994. The human gastric pathogen *Helicobacter pylori* has a gene encoding an enzyme first classified as a mucinase in *Vibrio cholerae*. Mol Microbiol **13:**153-60.

182. **Solnick, J. V., and P. Vandamme.** 2001. Taxonomy of the *Helicobacter* genus, p. 39-52. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* Physiology and Genetics. ASM Press, Washington, DC.

183. **Steinbach, G., R. Ford, G. Glober, D. Sample, F. B. Hagemeister, P. M. Lynch, P. W. McLaughlin, M. A. Rodriguez, J. E. Romaguera, A. H. Sarris, A. Younes, R. Luthra, J. T. Manning, C. M. Johnson, S. Lahoti, Y. Shen, J. E. Lee, R. J. Winn, R. M. Genta, D. Y. Graham, and F. F. Cabanillas.** 1999. Antibiotic treatment of gastric lymphoma of mucosa-associated lymphoid tissue. An uncontrolled trial. Ann Intern Med **131:**88-95.

184. **Stoebner, J. A., and S. M. Payne.** 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infect Immun **56:**2891-5.

185. **Storz, G., J. A. Opdyke, and A. Zhang.** 2004. Controlling mRNA stability and translation with small, noncoding RNAs. Curr Opin Microbiol **7:**140-4.

186. **Strachan, D. P.** 1989. Hay fever, hygiene, and household size. BMJ **299:**1259-60.

187. **Suerbaum, S., and M. Achtman.** 2001. Population Genetics, p. 355-362. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobater pylori* Physiology and Genetics. ASM Press, Washington, DC.

188. **Suerbaum, S., J. M. Smith, K. Bapumia, G. Morelli, N. H. Smith, E. Kunstmann, I. Dyrek, and M. Achtman.** 1998. Free recombination within *Helicobacter pylori*. Proc Natl Acad Sci U S A **95:**12619-24.

189. **Swisher, S. C., and A. J. Barbati.** 2007. *Helicobacter pylori* strikes again: gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Gastroenterol Nurs **30:**348-54; quiz 355-6.

190. **Sword, C. P.** 1966. Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. J Bacteriol **92:**536-42.

191. **Talley, N. J., A. R. Zinsmeister, A. Weaver, E. P. DiMagno, H. A. Carpenter, G. I. Perez-Perez, and M. J. Blaser.** 1991. Gastric adenocarcinoma and *Helicobacter pylori* infection. J Natl Cancer Inst **83:**1734-9.

192. **Tardat, B., and D. Touati.** 1993. Iron and oxygen regulation of *Escherichia coli* MnSOD expression: competition between the global regulators Fur and ArcA for binding to DNA. Mol Microbiol **9:**53-63.

193. **Telford, J. L., P. Ghiara, M. Dell'Orco, M. Comanducci, D. Burroni, M. Bugnoli, M. F. Tecce, S. Censini, A. Covacci, Z. Xiang, and et al.** 1994. Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. J Exp Med **179:**1653-58.

194. **Terebiznik, M. R., D. Raju, C. L. Vazquez, K. Torbricki, R. Kulkarni, S. R. Blanke, T. Yoshimori, M. I. Colombo, and N. L. Jones.** 2009. Effect of *Helicobacter pylori*'s vacuolating cytotoxin on the autophagy pathway in gastric epithelial cells. Autophagy **5:**370-9.

195. **Tielemans, C. L., C. M. Lenclud, R. Wens, F. E. Collart, and M. Dratwa.** 1989. Critical role of iron overload in the increased susceptibility of haemodialysis patients to bacterial infections. Beneficial effects of desferrioxamine. Nephrol Dial Transplant **4:**883-7.

196. **Tindberg, Y., C. Bengtsson, F. Granath, M. Blennow, O. Nyren, and M. Granstrom.** 2001. *Helicobacter pylori* infection in Swedish school children: lack of evidence of child-to-child transmission outside the family. Gastroenterology **121:**310-6.

197. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

198. **Tsutsumi, R., A. Takahashi, T. Azuma, H. Higashi, and M. Hatakeyama.** 2006. Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with *Helicobacter pylori* CagA. Mol Cell Biol **26:**261-76.

199. **van Vliet, A. H., J. Stoof, S. W. Poppelaars, S. Bereswill, G. Homuth, M. Kist, E. J. Kuipers, and J. G. Kusters.** 2003. Differential regulation of amidase- and formamidase-mediated ammonia production by the *Helicobacter pylori* Fur repressor. J Biol Chem **278:**9052-7.

200. **van Vliet, A. H., J. Stoof, R. Vlasblom, S. A. Wainwright, N. J. Hughes, D. J. Kelly, S. Bereswill, J. J. Bijlsma, T. Hoogenboezem, C. M. Vandenbroucke-Grauls, M. Kist, E. J. Kuipers, and J. G. Kusters.** 2002. The role of the Ferric Uptake Regulator (Fur) in regulation of *Helicobacter pylori* iron uptake. Helicobacter **7:**237-44.

201. **Wang, F., S. Cheng, K. Sun, and L. Sun.** 2008. Molecular analysis of the *fur* (ferric uptake regulator) gene of a pathogenic *Edwardsiella tarda* strain. J Microbiol **46:**350-5.

202. **Warren, J. R., and B. Marshall.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **1:**1273-5.

203. **Webster, A. C., and C. M. Litwin.** 2000. Cloning and characterization of *vuuA*, a gene encoding the *Vibrio vulnificus* ferric vulnibactin receptor. Infect Immun **68:**526-34.

204. **Weinberg, E. D.** 1978. Iron and infection. Microbiol Rev **42:**45-66.

205. **Weinberg, E. D.** 1974. Iron and susceptibility to infectious disease. Science **184:**952-6.

206. **Wilderman, P. J., N. A. Sowa, D. J. FitzGerald, P. C. FitzGerald, S. Gottesman, U. A. Ochsner, and M. L. Vasil.** 2004. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc Natl Acad Sci U S A **101:**9792-7.

207. **Wright, A. C., L. M. Simpson, and J. D. Oliver.** 1981. Role of iron in the pathogenesis of *Vibrio vulnificus* infections. Infect Immun **34:**503-7.

208. **Xiao, B., W. Li, G. Guo, B. Li, Z. Liu, K. Jia, Y. Guo, X. Mao, and Q. Zou.** 2009. Identification of Small Noncoding RNAs in *Helicobacter pylori* by a Bioinformatics-Based Approach. Curr Microbiol.

209. **Xiao, B., W. Li, G. Guo, B. S. Li, Z. Liu, B. Tang, X. H. Mao, and Q. M. Zou.** 2008. Screening and identification of natural antisense transcripts in *Helicobacter pylori* by a novel approach based on RNase I protection assay. Mol Biol Rep.

210. **Xiong, A., V. K. Singh, G. Cabrera, and R. K. Jayaswal.** 2000. Molecular characterization of the ferric-uptake regulator, Fur, from *Staphylococcus aureus*. Microbiology **146 (Pt 3):**659-68.

211. **Yokota, S., M. Konno, E. Mino, K. Sato, M. Takahashi, and N. Fujii.** 2008. Enhanced Fe ion-uptake activity in *Helicobacter pylori* strains isolated from patients with iron-deficiency anemia. Clin Infect Dis **46:**e31-3.

212. **Zahrt, T. C., J. Song, J. Siple, and V. Deretic.** 2001. Mycobacterial FurA is a negative regulator of catalase-peroxidase gene katG. Mol Microbiol **39:**1174-85.

Chapter Two

Expanding the Helicobacter pylori *Genetic Toolbox: a Modified, Endogenous Plasmid for Use as a Transcriptional Reporter and Complementation Vector*

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The work presented in this chapter is the sole work of B.M. Carpenter with the following exceptions: T.K. McDaniel constructed pTM117, J.M. Whitmire performed plasmid stability studies and generated the figures, H. Gancz created pDSM368 and DSM369, S. Guidotti and S. Censini isolated pHP666, D.S. Merrell performed Southern Blot analyses and plasmid stability studies.

Abstract

Helicobacter pylori is an important human pathogen. However, the study of this organism is often limited by a relative shortage of genetic tools. In an effort to expand the methods available for genetic study, an endogenous *H. pylori* plasmid was modified for use as a transcriptional reporter and as a complementation vector. This modification was accomplished by the addition of an *Escherichia coli* origin of replication, a

kanamycin resistance cassette, a promoterless *gfpmut3* gene, and a functional multiple cloning site to form pTM117. The promoters of *amiE* and *pfr*, two well characterized Fur-regulated promoters, were fused to the promoterless *gfpmut3,* and GFP expression of these fusions was analyzed by flow cytometry in wild-type and Δ*fur* strains under iron replete and deplete conditions. GFP expression was altered as expected based on current knowledge of Fur regulation of these promoters. RNase protection assays were used to determine the capacity of this plasmid to serve as a complementation vector by analyzing *amiE*, *pfr*, and *fur* expression in wild-type and Δ*fur* strains carrying a wild-type copy of *fur* on the plasmid. Proper regulation of these genes was restored in the Δ*fur* background under high and low iron conditions signifying complementation of both iron-bound and *apo* Fur regulation. These studies show the potential of pTM117 as a molecular tool for genetic analysis of *H. pylori*.

Introduction

Helicobacter pylori is a microaerophilic, Gram negative bacterium that causes diseases such as gastritis, peptic ulcer disease, MALT lymphoma, and gastric adenocarcinoma. While infection is chronic and often asymptomatic, the bacterium infects over 50% of the world's population (16). The sheer number of infected individuals leads to a significant number of *H. pylori*- associated disease cases. Moreover, since colonization usually occurs early in childhood and remains throughout the person's life unless treated (6), the chronicity of the infection increases the likelihood that disease will occur.

Part of the reason for *H. pylori's* success as a pathogen is due to the fact that the bacterium is well adapted for life in the gastric environment and produces a number of factors that facilitate its survival (16, 41). One of these factors is the ferric uptake regulator (Fur) (3), which has been shown to be important for colonization in the murine and gerbil models of *H. pylori* infection (7, 20). Fur, which acts as a transcriptional regulator, is crucial for iron homeostasis. While iron is essential for virtually all life forms, too much iron can lead to DNA and cellular damage by interacting with free oxygen to form hydroxyl radicals. Due to the complexities of iron homeostasis, the regulation of iron uptake and storage is crucial for the bacterium. Thus, a critical junction in the host-pathogen interaction is the former's attempt to sequester iron from the bacteria, and the latter's attempt to acquire iron from the host.

Fur functions in *H. pylori* in much the same way as it does in other bacteria; it binds to specific regions of iron-regulated promoters called "Fur boxes" and represses gene expression when it is itself bound by iron (Fe^{2+}), an indication of sufficient levels of cellular iron. Not surprisingly, genes regulated in this manner are often involved in iron acquisition. Repression of their expression prevents the deleterious effects of acquiring too much iron. In addition, Fur in *H. pylori* is known to down regulate another set of genes when in its *apo* form, i.e. Fur is not bound to its iron cofactor. This type of regulation occurs under iron deplete conditions and often involves the down regulation of iron storage genes (15). *apo* Fur regulation has not been identified in any other bacterial species making the study of Fur in *H. pylori* of particular interest. Also, Fur is implicated in the regulation of genes whose functions have no clear link to iron homeostasis and

plays a role in acid resistance (5, 7, 17, 20, 42); thus, further emphasizing Fur's global role in stress adaptation and its contribution to the success of *H. pylori* as a pathogen.

To date, the study of *H. pylori* has been somewhat limited by the relative lack of genetic tools for the study of this bacterium. For instance, while it is possible to make mutations in many strains of *H. pylori* by allelic replacement (2, 8) and transposon mutagenesis (18, 22, 34), it is often difficult to complement these mutations due to a lack of vectors that efficiently replicate and are stably maintained in the bacterium. One advance in this area occurred when pHP489, a cryptic *H. pylori* plasmid, was modified for use as a *H. pylori-E. coli* shuttle-vector for the genetic analysis of *H. pylori* (30). In that work, the authors describe the modification of pHP489 for this purpose and detail its use as a stable complementation vector (30). This was the first reported shuttle vector for use in the study of *H. pylori,* and pHP489 was shown to be useful as a complementation vector (30), though to our knowledge, there are no other reports in the literature of its utilization.

Another cryptic plasmid, pHel1, was also modified for use as a genetic tool in this organism (25, 26). This plasmid was modified to create pHel2 and pHel3 (26). In that study, these plasmids were shown to replicate autonomously in *H. pylori*. Moreover, the pHel derivatives were presented as useful complementation vectors, though the authors did note that not all attempts at complementation had been successful with the pHel vector system (26). Perhaps, in keeping with this limitation, these plasmids have currently not found wide use as complementation vectors in the *H. pylori* field; instead, many investigators currently achieve complementation by expression of their gene of interest from a non-native locus within the chromosome. One such popular system

described for *Helicobacter* involves allelic complementation in the *rdxA* locus, which when disrupted confers metronidazole (Mtz) resistance to the bacteria (11, 36). Since *rdxA* is responsible for the conversion of the non-toxic Mtz to the toxic hydroxylamine, *rdxA* mutation results in the inability to convert the non-toxic drug into the toxic by-product (21, 44). In addition to the *rdxA* system, another system for chromosomally-based complementation has recently been described by Langford, et al (28). In this system, complementation is achieved by inserting the gene of interest into the intergenic region between genes HP0203 and HP0204 through the use of a suicide plasmid vector containing the intergenic flanking regions, a MCS, and a chloramphenicol resistance cassette (28). Despite the definite advances these tools have provided to the *H. pylori* field, the number of complementation strategies available for the organism still lags behind in comparison to many other model organisms.

In addition to the relative lack of systems for complementation, there are currently no convenient systems to allow creation of transcriptional fusions in *H. pylori*. While *lacZ* transcriptional fusions have been shown to function in *H. pylori* (4, 12, 23), these studies required plasmid integration into the bacterial chromosome, thus creating the concern that the integration may cause polar effects. In addition to *lacZ*, GFP has also been used to monitor gene expression in *H. pylori* (27). In that study, Josenhans, et al. detail transcriptional promoter fusions to GFP that were integrated into the bacterial chromosome, requiring complicated strategies for cloning and integration and once again creating the possibility of polar effects. Additionally, these authors describe expression of GFP from the pHel2 (27) vector to create fluorescent "marker strains."

In light of the limited number of genetic tools that are available for the study of *H. pylori* as compared to other bacterial pathogens, there is a definite need to develop more vectors and methods for investigation of this important pathogen. We therefore describe the modification of another endogenous *H. pylori* plasmid, pHP666 (45), for use as both a GFP reporter plasmid and as a complementation vector. As a proof of principle, we show that the new system can be used to complement a *H. pylori fur* mutation and to study iron regulation by flow cytometry.

Material and Methods

Bacterial strains and growth.

All strains and plasmids used in this study are listed in Table 2, and primer sequences are listed in Table 3. *H. pylori* strains were maintained as frozen stocks in brain heart infusion medium supplemented with 20% glycerol and 10% fetal bovine serum (FBS) (Gibco) at -80°C. Bacterial strains were grown on horse blood agar (HBA) plates made of 4% Columbia agar base (EMD Chemicals, Inc.), 5% defibrinated horse blood (HemoStat Laboratories, Dixon, CA), 0.2% β-cyclodextrin (Sigma), 10μg/mL vancomycin (Amresco), 5μg/mL cefsulodin (Sigma), 2.5U/mL polymyxin B (Sigma), 5μg/mL trimethoprim (Sigma), and 8μg/mL amphotericin B (Amresco). *H. pylori* liquid cultures were grown shaking at 100 rpm in brucella broth supplemented with 10% FBS and 10μg/mL vancomycin at 37°C. Where noted in Table 2, plates and cultures were supplemented with 25μg/mL kanamycin (Kan) (Gibco) and/or, 25μg/mL or 8μg/mL chloramphenicol (Cm) (EMD Chemicals, Inc.). Both plate and liquid cultures were

Table 2. Plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pHP666	endogenous *H. pylori* plasmid isolated from CCUG 17874	(45)
pHel2	*E. coli-H. pylori* shuttle vector	(26)
pTM117	Modified pHP666 to include *E. coli* origin of replication, *aphA-3* cassette (Kan^R), multiple cloning site, and promoterless *gfpmut3*	this study
pDSM221	pTM117 *amiE* promoter::*gfpmut3* fusion	this study
pDSM368	pTM117 *pfr* promoter::*gfpmut3* fusion	this study
pDSM227	*fur*::pHel2 complementation vector	this study
pDSM340	*fur*::pTM117 complementation vector	this study
H. pylori strains		
G27	WT *Helicobacter pylori*	(10)
DSM145	G27 Δ*fur*::*aphA-3*, Kan^R (25μg/mL)	(20)
DSM300	G27 Δ*fur*::*cat*, Cm^R (25μg/mL or 8μg/mL)	this study
DSM215	G27 (pTM117), Kan^R (25μg/mL)	this study
DSM235	G27 (pDSM221), Kan^R (25μg/mL)	this study
DSM305	DSM300 (pDSM221), Kan^R, Cm^R (25μg/mL each)	this study
DSM369	G27 (pDSM368), Kan^R (25μg/mL)	this study
DSM370	DSM300 (pDSM368), Kan^R, Cm^R (25μg/mL each)	this study
DSM279	G27 (pDSM227), Cm^R (8μg/mL)	this study
DSM281	DSM145 (pDSM227), Kan^R, Cm^R (25μg/mL, 8μg/mL)	this study
DSM341	G27 (pDSM340), Kan^R (25μg/mL)	this study
DSM343	DSM300 (pDSM340), Kan^R, Cm^R (25μg/mL, 8μg/mL)	this study

grown in gas evacuation jars under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) generated by an Anoxomat gas evacuation and replacement system (Spiral Biotech).

All *H. pylori* strains in this work are derivatives of G27 (10). Two isogenic *fur* (HP1027) mutants were utilized in this study--strains DSM145 (Δ*fur*1) and DSM300 (Δ*fur*2). DSM145 was described previously and contains a deletion insertion of the *fur* coding sequence with the *aphA-3* gene (conferring Kan resistance) from *Campylobacter coli* (20). To create DSM300, the *aphA-3* gene was removed from the original pΔHP1027-K7 suicide vector and was replaced with the *chloramphenicol acetyltransferase* (*cat*) gene from *C. coli*. This was accomplished by digestion of pΔHP1027-K7 with ClaI (Invitrogen), to remove the *aphA-3* gene, followed by replacement with the NarI (New England Biolabs) digested *cat* gene. The *cat* gene had been amplified from the pGPS-cat vector (34) with primers catF-nar and catR-nar, which contain NarI restriction sites. NarI and ClaI have compatible sticky ends, and thus, their digestion products are able to be ligated. This ΔHP1027::*cat* construct was then naturally transformed into WT G27 to generate DSM300.

pTM117 construction

pHP666, an endogenous *H. pylori* plasmid (45), was used as the backbone for pTM117. Though not specifically named in the work of Xiang, et al., pHP666 was isolated from the 1995 study by these authors (45). The entire pHP666 sequence was amplified by PCR with primers F666NotI and R666NotI, which incorporate unique NotI sites for subsequent cloning steps. To facilitate replication of the plasmid in *E. coli,* the origin of replication and the *rop* gene from pBR322 (35) were amplified with primers

BR322-F (PstI, HpaI) and BR322-R (NotI), which incorporate the indicated restriction sites. The *aphA-3* gene from *C. coli* was also amplified using primers aphA-F (XbaI, BamHI, SmaI, KpnI, and SacII) and aphA-R (NotI) from pIP1433 (39). The aphA-F primer was designed to incorporate a multiple cloning site (MCS) into its fragment, which eventually would generate the MCS for pTM117. Finally, primers GF1 (XbaI) and GFP-R (PstI) were used to amplify a promoterless green fluorescent protein mutant3 (*gfpmut3*) derivative (9). Each resulting PCR fragment was restriction digested according to standard procedures as follows: pHP666 – NotI (New England Biolabs), *E. coli* origin of replication– PstI (Invitrogen)/NotI, *aphA3* – XbaI (Invitrogen)/NotI, and *gfpmut3* – XbaI/PstI. The fragments were all gel purified using the Qiagen Gel Purification Kit and subsequently joined in a four part ligation. The resulting ligation products were transformed into *E. coli* DH5α, and transformants were selected for on Kan. Plasmid isolates were restriction digested to confirm the presence of all four fragments, and the construct was named pTM117. This plasmid was introduced into *H. pylori* via natural transformation with 1-1.5μg of plasmid DNA as described (33) and transformants selected for on HBA plates containing Kan. Strain DSM215 is wild-type (WT) G27 *H. pylori* bearing pTM117.

Sequencing of pTM117

To verify the full sequence of pTM117, the plasmid was sequenced with each of the primers listed in Table 3. Sequencing reactions were performed using BigDye Terminator 3.0 reagent (Applied Biosystems, Inc.) according to the manufacturer's directions with 345ng of pTM117 and 30pmol of primer (1μL at 30μM). The sequencing

reactions ran for 25 cycles with cycling as follows: 96°C for 30seconds (sec.), 50°C for 15sec., 60°C for 4 minutes (min.). Sequencing reactions were cleaned using Performa DTR Gel Filtration Cartridges (Edge Biosystems) and an Eppendorf Centrifuge 5415D; columns were spun for 2min. at 1.5xg, samples added, and eluted for 1min at the same speed. The reactions were run on a Genetic Analyzer 3130XL (Applied Biosystems, Inc.) and analyzed using Invitrogen Vector NTI 10.0 software. Each sequencing reaction for each primer was independently performed three times, and all sequencing results were assembled to generate a 3x pass of the final vector sequence of pTM117 (Genbank accession no. EF540942).

Creation of promoter fusions

Transcriptional fusions of *amiE* (HP0294) and *pfr* (HP0653) to the promoterless *gfpmut3* in pTM117 were constructed by amplification of the promoters of each gene using primer pairs amiE-F and amiE-R (319bp product) and HP0653_Promoter_F and HP0653_Promoter_R (305bp product), respectively. These primers incorporate SacII and BamHI restriction sites on the 5' and 3' ends, respectively, to facilitate directional ligation into pTM117. Each promoter fragment was subcloned into pGEM T-Easy (Promega), digested with SacII (New England Biolabs) and BamHI (Invitrogen), and ligated to the appropriately digested pTM117 to create pDSM221 (*amiE*::*gfpmut3*) and pDSM368 (*pfr*::*gfpmut3*). Each promoter fusion was confirmed by sequencing with the apha3-2 primer (Genbank accession nos. *amiE* promoter - EF537053 and *pfr* promoter - EF537052). These plasmids were moved into WT G27 and DSM300 by natural transformation, and transformants were selected on plates containing 25μg/mL Kan and

Table 3. Primers used in this study

Primer	Sequence (5'-3')*	Reference
pTM117 construction primers		
F666NotI	*ATAAGAAT*GCGGCCGCAGAGATTGAAACAGACTATTTAGAAAA	this study
R666NotI	*ATAAGAAT*GCGGCCGCTCATTATATTTTCTAACTCACTCTCTC	this study
BR322-F(PstI, HpaI)	*AAAA*CTGCAGTTAACTCCCGCCGCATCCATACCGCC	this study
BR322-R (NotI)	*ATAAGAAT*GCGGCCGCCACTGAGCGTCAGACCCCGTA	this study
AphA-F (XbaI, BamHI, SmaI, KpnI, SacII)	*GATT*TCTAGAGGATCCCCGGGTACCGCGGTCGATACTATGTTATACGCC	this study
AphA-R (NotI)	*ATAAGAAT*GCGGCCGCAGACATCTAAATCTAGGTACTA	this study
GF1 (XbaI)	*GATT*TCTAGAATTTAAGAAGGAGATATACATATGAGTAAAGGAGAAG	(9)
GFP-R (PstI)	*AAAA*CTGCAGGTCTGGACATTTATTTGTATAG	this study
Sequencing primers		
gfp-1	AAGTCGTGCTGCTTCATGTG	this study
gfp-F1	CCTGTTCCATGGCCAACACTTG	this study
gfp-F1a	CCAGACCTGCAGTTAACTC	this study
gfp-F2	CCACGCTGATGAGCTTTAC	this study
gfp-F3	GGTAAGACACGACTTATCG	this study
gfp-F4	GCTTTCAAGCAAGTAATTC	this study
apha3	GAAAGAGCCTGATGCACTCC	this study
apha3-2	CGGTGATATTCTCATTTTAGCC	this study
apha3-R1	GGCTGGAGCAATCTGCTCATG	this study
apha3-R2	GGAAGAACAGTATGTCGAG	this study
apha3-R2a	CGTGTTCTTGCATAAAGGTTG	this study
apha3-R3	GCTCTCTTTCAAGTTCAAGG	this study
BB-F1	GCGGTGTTGTCAATCTGC	this study

BB-F2	GCTAGGTTTGTTCAATTC	this study
BB-F3	CGGTATAATAGTGCAAGTC	this study
BB-F4	GGTTTGCGCTATCTTGTTTG	this study
BB-F5	CCGTATAATGCTTAAAGAC	this study
BB-R1	GGAGCTGTTGATATTTCTC	this study
BB-R2	GGATAAGCACCCTACACATG	this study
BB-R3	CCGTTCTTTGTAGCTACAC	this study
BB-R4	GGTTGCTTAGGTTAGGTAG	this study
BB-R5	CCACTCAGGAAATTTGAG	this study
Promoter::*gfp* fusion primers		
amiE-F (SacII)	CCGCGGGCATGCACCTTTGAAATTGC	this study
amiE-R (BamHI)	GGATCCGCTGCTGCTACTAATATCTCCATG	this study
HP0653_Promoter_F (SacII)	CCGCGGTGGTTAAATTGCCCTTTCGT	this study
HP0653_Promoter_R (BamHI)	GGATCCGATAACATAGTATCTCCTTTGTGTTGG	this study
Complementation primers		
FurCF (XbaI)	TCTAGAAAGGCTCACTCTACCCTATT	this study
FurCR (SalI)	GTCGACAAGACTTTCACCTGGAAACGC	this study
RPA primers		
amiE-RPA-F	GGTTTGCCTGGGTTGGAT	(20)
amiE-RPA-R	GATTTTGCGGTATTTTG	(20)
pfr-RPA-F	GCGGCTGAAGAATACGAG	this study
pfr-RPA-R	CTGATCAGCCAAATACAA	this study
fur-RPA-F	GAGCGCTTGAGGATGTCTATC	this study
fur-RPA-R	GTGATCATGGTGTTCTTTAGC	this study

[*]Restriction endonuclease sites are underlined, and linker bases are italicized.

25μg/mL Kan plus 25μg/mL Cm, respectively. pDSM221 in WT G27 is strain DSM235, and pDSM221 in DSM300 is strain DSM305. Likewise, pDSM368 in WT G27 is strain DSM369 and in DSM300 is strain DSM370.

GFP expression reporter assays

Flow cytometry was utilized to assess the ability of the *amiE* and *pfr* promoters to drive the expression of GFP in their corresponding plasmid constructs. Strains DSM235 and DSM369 were grown for 48 hours in liquid culture as described above with and without 60μM 2, 2'-dipyridyl (dpp) (Sigma), an iron chelator. This is a sufficient concentration of chelator to slow but not severely hinder bacterial growth. For comparison, DSM305 and DSM370 were grown for 48 in liquid culture media without dpp. 1.5mL of each culture was pelleted and resuspended in 1x sterile phosphate buffered saline (PBS). The resuspensions were then passed through a 1.2μm Acrodisc PSF Syringe filter (Pall) to remove any bacterial clumps or debris. The samples were then analyzed using a Beckman Coulter Epics' XL-MCL Flow Cytometer. The laser was set at 750Volts, and the instrument collected 100K events. Flow cytometry data was analyzed using WinList 3D ver. 6.0 (Verity Software House).

Creation of Fur complementation vectors

A 923bp WT copy of the *fur* gene (HP1027) was amplified with primers FurCF (XbaI) and FurCR (SalI) and subcloned into pGEM T-Easy. This product encompasses the entire Fur coding sequence as well as the predicted promoter region (14). The pHel2

complementation vector was constructed by liberating the *fur* gene from pGEM T-easy by SalI (New England Biolabs) digestion (there is a SalI site within the vector's backbone). pHel2 was similarly digested, and treated with calf intestinal phosphatase (New England Biolabs) to prevent pHel2 self-ligation (26). The SalI digested *fur* fragment was then ligated to pHel2 to create pDSM227. The resulting plasmid was transformed into WT G27 and DSM145 (ΔHP1027, Kan^R). These transformations yielded strains, DSM279 and DSM281, respectively. Additionally, the *fur* gene was liberated from pGEM T-Easy by digestion with PstI and SacII (sites inherent to the pGEM T-easy vector multi-cloning site) and was ligated to the appropriately digested pTM117 generating pDSM340 (digestion of pTM117 with PstI and SacII results in the removal of the promoterless *gfpmut3* gene). pDSM340 was sequenced with the FurCR (SalI) primer to confirm the correct construct. pDSM340 was then transformed into WT G27 and DSM300, and transformants were selected for on the appropriate antibiotics (see Table 2). DSM341 (pDSM340 in WT G27) and DSM343 (pDSM340 in DSM300) are the strains resulting from these transformations.

RPAs

RNase protection assays (RPAs) were used to assess the ability of pDSM340 and pDSM227 to complement a *fur* deletion. G27, DSM145, DSM300, DSM341, DSM343, DSM279, and DSM281 were grown overnight in liquid culture with the appropriate antibiotics to maintain selection for the plasmids. The pHel2 *fur* complementation strains, DSM279 and DSM281 were grown with 8μg/mL Cm, and with 8μg/mL Cm plus 25μg/mL Kan, respectively. The pTM117 *fur* complementation strains, DSM341 and

DSM343, were grown with 25µg/mL Kan and 25µg/mL Kan plus 8µg/mL Cm, respectively. Half of the culture was removed for RNA isolation (t_0), while the other half was exposed to 200µM dpp (t_{60}) for one hour. The addition of 200µM dpp causes rapid chelation of the iron from the media and results in an iron deplete environment (31). RNA was extracted as previously described (37), and 1.5 – 2.0µg of RNA was used in each RPA reaction. Riboprobe templates for *amiE, pfr*, and *fur* were generated by PCR using the primer pairs listed in Table 3. The templates were ligated to pGEM T-easy, and their orientation was subsequently determined by PCR. Probes were generated using a Maxiscript kit (Promega) and 50µCi [^{32}P]UTP (Perkin-Elmer), and RPAs were performed with the RPA III kit (Ambion) as previously described (20). RPA reactions were resolved on 5% acrylamide-1x Tris-Borate-EDTA-8M urea denaturing gels, and the gels were exposed to Kodak phosphor screens. These phosphor screens were scanned using a FLA-5100 Multifunctional Scanner (Fujifilm) and analyzed using ImageGauge Ver. 4.22 software (Fujifilm).

Plasmid copy number

Southern Blot analysis of total DNA was used to determine plasmid copy number as previously described (26). Briefly, total genomic DNA was isolated from WT G27, DSM341, and DSM343 using the Easy-DNA kit (Invitrogen). The DNA was digested with NotI or NotI and HindIII, which liberates the *fur* gene from pTM117 and infrequently cuts the chromosomal DNA. Southern blots were performed using the ECL Direct Nucleic Acid Labeling and Detection System kit (Amersham Biosciences) according to standard procedures, probing with end-labeled *fur*. The relative number of

copies of *fur* present between WT G27, DSM341, and DSM343 was determined using a FLA-5100 Multifunctional Scanner (Fujifilm) and ImageGauge Ver. 4.22 software (Fujifilm). In addition, this experiment was repeated using WT G27, DSM235, and DSM305 total genomic DNA, cut with NotI, and probing for the *amiE* promoter.

Plasmid stability studies

Strain DSM215 was cultured overnight in liquid media supplemented with 25µg/mL Kan. These Day 0 cultures were used to inoculate Day 1 cultures in liquid media without Kan. The Day 1 cultures were grown overnight and subcultured into fresh liquid media without Kan at a 1:20 dilution. This re-inoculation cycle was repeated through the start of Day 5 liquid cultures. Samples of the cultures from Day 0, Day 1, Day 3, and Day 5 were plated on HBA plates to obtain single colonies. Four days later the colonies were replica plated onto a HBA plate and a HBA plate supplemented with 25µg/mL Kan. Two days after the replica plating, the HBA plates supplemented with Kan were compared to the unsupplemented HBA plates to identify any Kan sensitive colonies and assess the stability of the pTM117 plasmid upon repeated passages in liquid media in the absence of selection. This experiment was repeated as above using strains DSM341 and DSM343 (pTM117 *fur* complementation vector in WT G27 and Δ*fur* backgrounds, respectively) with daily re-inoculations out to the start of Day 3.

Nucleotide sequence accession numbers

The nucleotide sequences of pHP666, pTM117, the G27 *amiE* promoter, and the G27 *pfr* promoter can be found in the GenBank database using accession numbers DQ198799, EF540942, EF537053, and EF537052, respectively.

Results

Construction of pTM117

Since there is a relative deficit in genetic tools available for use in *H. pylori*, and since there is currently only one plasmid system that has gained popular use in this organism (26), we sought to create an additional system to expand the repertoire of available genetic tools. Since the pHel system (26) had been shown to work well in some, but not all *H. pylori* strains, we decided to adopt a similar strategy for developing our own system: modification of an endogenous *H. pylori* plasmid. However, to potentially expand the diversity of *H. pylori* strains in which the plasmid would be useful, we sought to use a plasmid backbone that was not too closely related to the pHel system. We had previously isolated and sequenced pHP666 from strain CCUG 17874 (45). Comparison of pHP666 to pHel1 (Genbank accession numbers DQ198799 and Z49272, respectively) revealed that the only significant conservation between the two plasmids was in the open reading frame predicted to encode RepA. This RepA replicase seems to be very well conserved among virtually all characterized *H. pylori* endogenous plasmids. Further analysis of the predicted origins of replication of pHP666 and pHel1 suggests that they are different since the iterons are not conserved. Given these distinct differences, we reasoned that the pHP666 backbone should serve as a good candidate for modification.

Our strategy was formulated with the idea that the resulting vector could be used to create transcriptional fusions to GFP as well as serve as a shuttle vector for complementation. To this end, we constructed pTM117 as described in the Materials and Methods section. Briefly, the vector contains the origin of replication from pBR322 (35), an *aphA-3* cassette from *C. coli* (39), and a promoterless *gfpmut3* allele (9). These factors allow for plasmid replication in *E. coli*, selection on Kan in *E. coli* and *H. pylori*, and the creation of transcriptional fusions to enhanced GFP to monitor promoter activity in *H. pylori*, respectively. The pTM117 vector map is shown in Fig. 3, and the complete sequence is available at Genbank accession number EF540942.

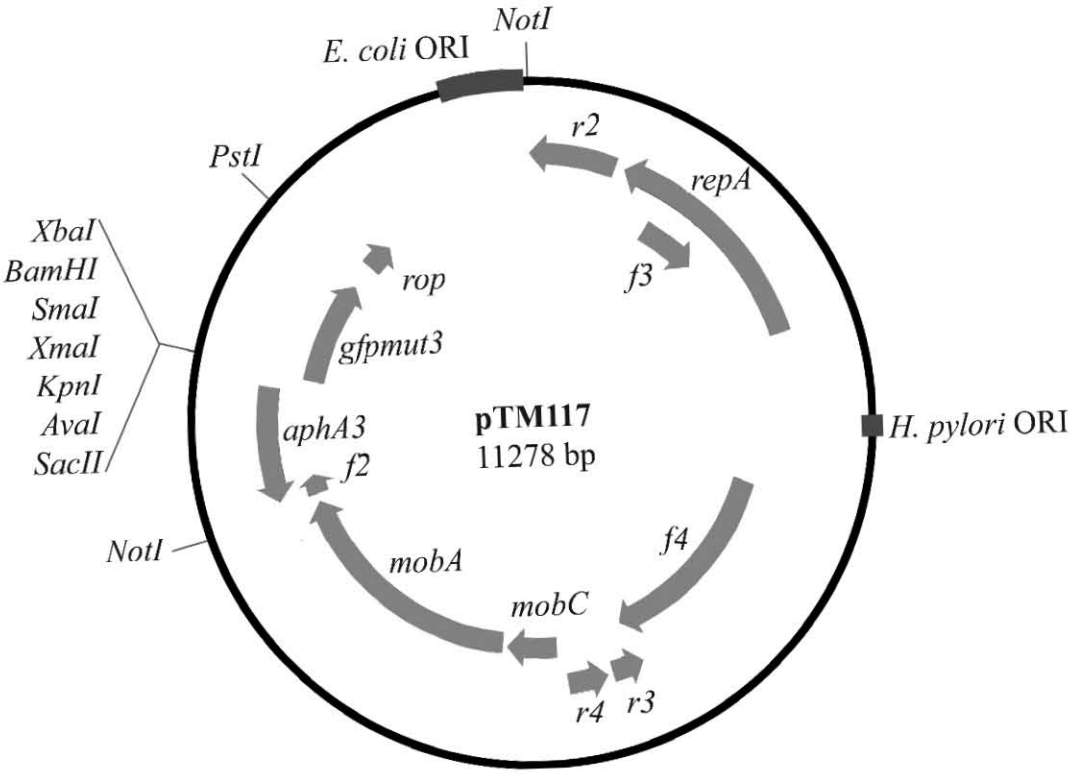
pTM117 GFP reporter assays

pTM117 was engineered with the idea that promoters of interest could easily be studied by transcriptionally fusing them to the promoterless *gfpmut3* gene within the vector. In this way, relative levels of fluorescence can be used as a reporter of promoter activity, and GFP expression can be monitored by flow cytometry. To determine if pTM117 could be utilized in this fashion, *amiE* and *pfr* promoter fusions were constructed. These promoters were chosen since a number of studies have shown that they are regulated by Fur in an inverse manner: *amiE* is repressed by iron-bound Fur (43), and *pfr* is repressed by *apo* Fur (15, 43). Though we were unable to detect GFP expression using a hand-held UV light, examination of *H. pylori* strains carrying either of these fusions by fluorescence microscopy confirmed detectable GFP fluorescence (data not shown). Additionally, flow cytometry showed distinct peaks of fluorescence, significantly higher than background (i.e. the fluorescence of an isogenic strain carrying a

Figure 3. Physical and genetic map of the E. coli – H. pylori *shuttle vector pTM117.*

Plasmid pHP666 composes the backbone of the pTM117 vector and contains the *H. pylori* origin of replication. Kanamycin resistance is encoded by the *aphA-3* gene, and a multi-cloning site lies upstream of the promoterless *gfpmut3* allele. Unique multi-cloning restriction enzyme sites are bracketed in the indicated area upstream of *gfpmut3*. Predicted open reading frames, with names of the closest gene homologues, are represented by grey arrows, and the direction of transcription is indicated by the direction of the individual arrows.

Figure 3. Physical and genetic map of the E. coli – H. pylori *shuttle vector pTM117.*



promoterless plasmid (Fig. 4 and data not shown). We next examined whether or not we could visualize iron and Fur-dependent regulation of *amiE* and *pfr*. Based on the literature, we would expect to see an increase in GFP expression driven by the *amiE* promoter (43) and a decrease in *pfr* promoter driven GFP expression (15) when bacteria are iron limited. Changes in GFP expression under the control of the promoter of *amiE* are shown in Fig. 4A, and alterations in GFP expression under the control of the *pfr* promoter are shown in Fig. 4B. As expected, *amiE* promoter-driven expression was increased by iron limitation while *pfr* promoter-driven expression was decreased. Moreover, both promoter fusions were deregulated when carried in a Δ*fur H. pylori* strain. These data suggest that pTM117 can be used to create transcriptional fusions to GFP, which can then be monitored by flow cytometry.

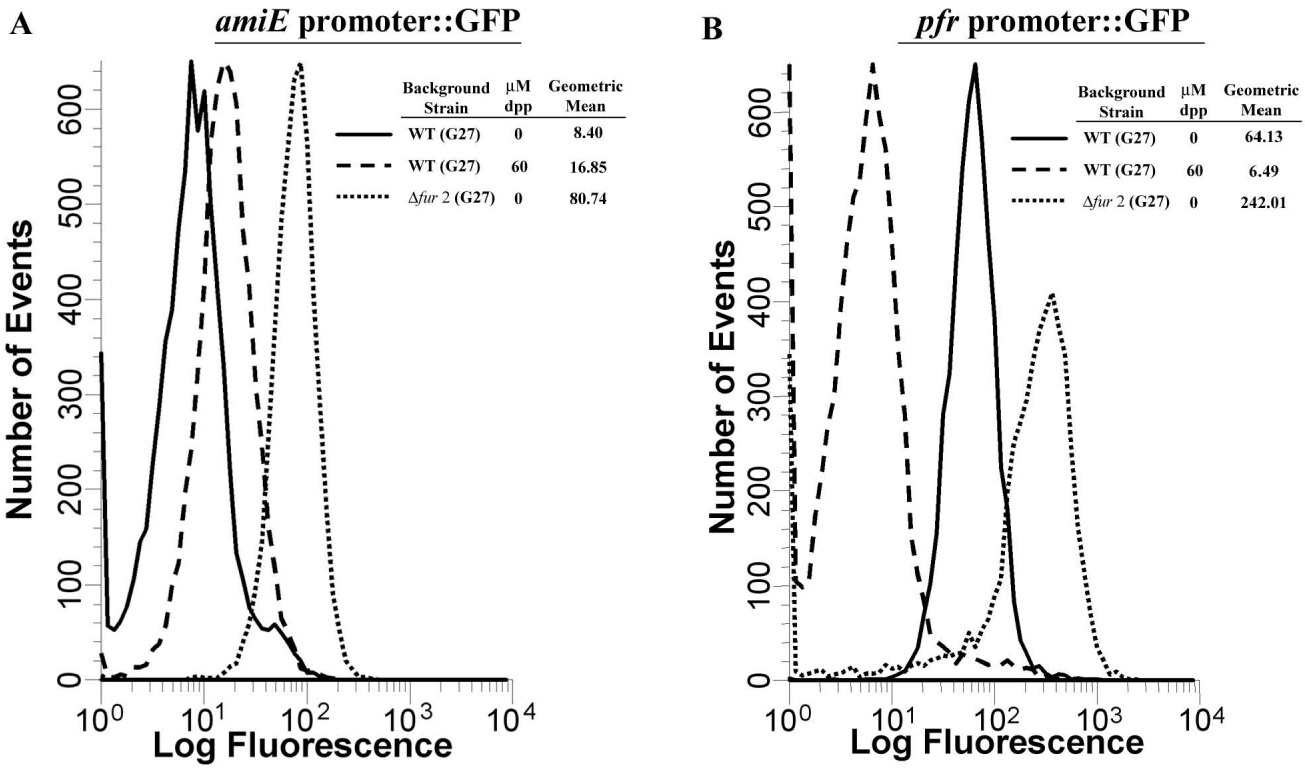
Utilization of pTM117 for complementation

Since complementation in trans is often difficult in *H. pylori*, we next assessed the ability of pTM117 to be used as a complementation vector. For these studies, we chose to examine complementation of a fur mutation. For comparison we concurrently constructed a fur complementation vector using pHel2 (26). RPAs were then used to assess the ability of both vectors to complement iron-bound and apo Fur regulation in Δfur G27. Once again, we monitored amiE and pfr expression as these genes are iron-bound and apo Fur regulated, respectively. We also analyzed fur expression in these strains to compare the basal level of fur expression in these strains under normal and iron chelated conditions and to assess the ability of the fur bearing vectors to complement the iron-bound autoregulation of Fur. RNA was isolated for each test (complemented) and

Figure 4. Flow cytometry analysis of pTM117 GFP transcriptional fusions.

Strains bearing *amiE*::*gfpmut3* or *pfr*::*gfpmut3* promoter fusions were grown in iron replete or deplete media for 48 hours and analyzed for fluorescence as described in the Material and Methods section. Panel 2A contains the results for the *amiE*::*gfpmut3* fusions, and panel 2B contains the results for the *pfr*::*gfpmut3* fusions. For both A and B, solid lines indicate the plasmid in WT *H. pylori* G27 grown in iron replete conditions, the dashed lines indicate the plasmid in WT bacteria grown in iron-deplete conditions, and the dotted lines indicate the plasmid in Δ*fur* bacteria grown in iron-replete conditions. Fluorescence is measured in relative units, and the data are representative of multiple independent flow analyses.

Figure 4. Flow cytometry analysis of pTM117 GFP transcriptional fusions.



control (WT and Δ*fur*) strain prior to and after an additional hour of exposure to 200μM 2,2'-dipyridyl (dpp). This concentration of dpp rapidly chelates the available iron and does not affect viability of the bacterial cells (31). As shown in Fig. 5 and as expected, the addition of dpp to WT bacteria resulted in a large increase in *amiE* expression (5.8-fold). This increase was not seen in the Δ*fur* strains. When WT bacteria carried an additional copy of the *fur* gene in the context of pHel2 or pTM117, we again saw a large increase in *amiE* expression upon exposure to dpp. Iron-bound Fur regulation of *amiE* was partially restored in the *fur* mutant carrying pHel2 (2.1-fold) and fully restored in the strain carrying pTM117 (6.2-fold). These data suggest that while either pHel2 or pTM117 can be used to complement iron-bound Fur regulation in a *fur* deficient strain, full complementation is only achieved with pTM117.

To determine if both vectors could also complement *apo* Fur regulation, we next monitored *pfr* expression. As expected, the addition of dpp to WT bacteria resulted in a decrease in *pfr* expression (0.3-fold) as shown is Fig. 5. Once again, this change in expression is not seen in the Δ*fur* mutants. WT bacteria carrying both complementation vectors exhibited the expected decrease in *pfr* expression upon iron chelation. Once again, *fur* carried on pHel2 showed partial complementation (0.5-fold) while the pTM117 derivative showed full complementation of *apo*- regulation (0.3-fold). In order to verify that there were no mutations in the pHel2 and pTM117 constructs that could account for the difference in the levels of complementation, we sequenced the *fur* insert from both vectors (data not shown). No mutations in either the promoter or coding sequence of *fur* in either vector were identified, thus complementation differences are not due to introduced mutations.

In order to assess the ability of the complementation vectors to complement *fur* autoregulation and to compare the basal levels of *fur* expression in each strain, RPAs were also performed using a *fur* riboprobe. The addition of dpp to WT bacteria resulted in a 2.7-fold increase in *fur* expression as shown in Fig. 5. This increase is what is expected based on current literature (13, 14). In the Δ*fur* background, pTM117 was able to restore *fur* regulation to a level similar to WT (2.3-fold), while *fur* complementation with pHel2 showed only a modest increase in *fur* expression under iron chelated conditions. This mirrors the trends seen with both *amiE* and *pfr*, i.e. that we achieved partial complementation with pHel2 and full complementation with pTM117 (Fig. 5). Taken together, the RPA data indicate that pTM117 can be used as an effective complementation vector.

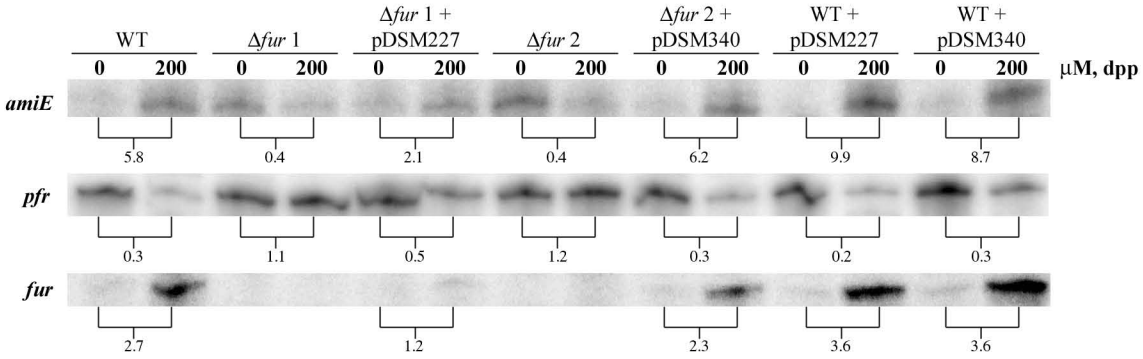
Plasmid characterization

To expand our understanding of pTM117 and its derivatives, we next examined some of the basic vector characteristics to determine copy number and plasmid stability. Determination of copy number was accomplished using Southern blot analysis to examine the relative copy number of *fur* found in the chromosome (one copy) as compared to on the plasmid in strains carrying the pTM117 *fur* complementation vector. Additionally, since we reasoned that the relative copy number of the plasmid could be artificially depressed if carrying too many copies of *fur* was deleterious to the bacteria, we examined the relative copy number of the *amiE* promoter found on the chromosome as compared to on the plasmids in strains carrying the *amiE*::*gfpmut3* transcriptional fusions. In each case, we determined that there were one to two copies of pTM117 per

Figure 5. Determination of the ability of pTM117 and pHel2 to complement iron-bound and apo-*Fur regulation.*

The indicated strains were grown overnight in iron replete liquid media. On the subsequent day, one half was used for RNA isolation. The other half was exposed to iron deplete conditions for one hour by the addition of 200μM dpp prior to isolation of the RNA. Top Panel: an *amiE* riboprobe was used to determine iron-bound Fur complementation. Middle Panel: a *pfr* riboprobe was used to quantitate *apo* Fur complementation. Bottom Panel: a *fur* riboprobe was used to quantitate Fur complementation. Fold-changes are indicated below each pair and were calculated by comparing the relative amount of protected riboprobe in the iron deplete environment (200μM dpp) to the iron replete lane (0μM dpp). These data are representative of multiple independent experiments.

Figure 5. Determination of the ability of pTM117 and pHel2 to complement iron-bound and apo-*Fur regulation.*



per bacterial cell (Fig. 6 and data not shown), suggesting that pTM117 is a very low copy number plasmid.

To determine plasmid stability, we serially passaged WT G27 carrying pTM117 for up to 5 days in the absence of antibiotic selection. The results of three independent experiments (A-C) are shown in Table 4. The percent of colonies that remained Kan resistant when grown in the absence of antibiotics for the indicated times are shown relative to the day zero starting inoculums. For each experiment, we consistently obtained greater than 99% stability for each passage. Similar stability rates were also found when using the pTM117 *fur* complementation plasmid in the WT G27 and the Δ*fur* backgrounds (data not shown). These data demonstrate that pTM117 and its derivatives are stable in the absence of antibiotic selection.

Finally, we investigated whether pTM117 remained episomal within the *H. pylori* cell. We reasoned that if pTM117 was integrated into the chromosome that we should be unable to recover plasmid from *H. pylori* strains bearing the pTM117 fusions. Therefore, we performed plasmid preps on *H. pylori* strains bearing pTM117 (empty vector) as well as those bearing the *pfr* and *amiE* reporter fusions, and the *fur* complementation vector (from both WT and Δ*fur* G27). The resultant DNA was then used to transform *E. coli*. In each case we recovered Kan resistant *E. coli* strains that bore the pTM117 plasmid derivatives – showing that pTM117 does remain episomal in the *H. pylori* cell. These data are further supported by the fact that we obtained the expected size bands for a chromosomal and plasmid born fragment in the Southern blot data (Fig. 6).

Figure 6. Southern blot to determine plasmid copy number.

Total genomic DNA was isolated from the indicated strains, digested with NotI and HindIII, and subjected to Southern blot analysis with an end-labeled *fur* probe. The top band represents the chromosomal copy of *fur* while the bottom band represents the plasmid born copy of the gene. These data are representative of multiple independent experiments.

Figure 6. Southern blot to determine plasmid copy number.

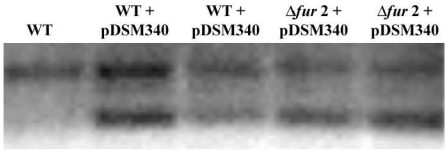


Table 4. Percent H. pylori *KanR CFU in the absence of Kan selection* *

Trial	Day 1	Day 3	Day 5
A	99.87	99.30	99.73
B	100	99.71	100
C	99.98	99.97	ND[+]

[*]Data represent three independent experiments and indicate the percent of Kan^R colonies out of the total number of colonies on the indicated day. [+]ND indicates the day was not determined.

Discussion

Tools for the genetic study of *H. pylori* are few compared to what are available for many other bacteria, and given that *H. pylori* is the most common bacterial infection of man, there is a real need for continued study of this pathogen. Herein we describe the creation of pTM117, its use as a GFP transcriptional reporter, and its use as a complementation vector. We chose pHP666 (45) as the backbone for our system based on the previous successes at modification of cryptic plasmids for use in *H. pylori* (26, 30). Moreover, since pHP666 appears to be found in a diverse number of *H. pylori* strains (45), we reasoned that it might be broadly usable. pTM117 was therefore created by the addition of the *apha-3* gene for selection on Kan in *E. coli* and *H. pylori*, the promoterless *gfpmut3* allele for creation of transcriptional fusions to the enhanced GFP protein to monitor promoter activity in *H. pylori*, and an *E. coli* origin of replication to allow for replication of the plasmid in that organism. Basic characterization of pTM117 showed that it is a low copy number vector, which is stably maintained in G27.

Using two well-studied Fur regulated promoters, *amiE* and *pfr,* we show that pTM117 can be used as a GFP reporter plasmid. As our plasmid design incorporates a multi-cloning site upstream of the promoterless *gfpmut3,* in theory, virtually any promoter of interest could be cloned into pTM117 and GFP expression monitored as a reporter of transcriptional activity. While this technique has been broadly used in other bacteria, to our knowledge, this is the first time flow cytometry has been used to analyze GFP expression in *H. pylori*. Previously, GFP expression in *H. pylori* has been monitored by fluorescent microscopy (26) or by western blot (27). These previous studies expressed GFP from the *flaA* (27) or *flaB* (26) promoters in the context of pHel2

and from the *flaA* or *flaB* promoters by chromosomal integration into those loci (27). pTM117 is the first easily usable transcriptional reporter plasmid designed for use in *H. pylori*. We predict that this system can be used to monitor activity of most clonable *H. pylori* promoters, and should be able to be used for differential fluorescence induction (40) to monitor changes in expression of promoters of interest upon exposure to diverse environmental conditions. To this end, our laboratory has successfully made *cagA*, *vacA*, *ureA*, and *napA* fusions to *gfpmut3*. Moreover, DFI has been performed on strains carrying these reporters in an effort to identify regulators of these important virulence factors (K. Jones and D.S. Merrell unpublished data).

There are many advantages to using flow cytometry to study bacterial pathogenesis: mainly that flow cytometry is very efficient and allows for large numbers of samples to be analyzed in a relatively short period of time. Additionally, sample preparation is simple. For those not versed with flow cytometry or requiring alternative antibiotic selection, it is conceivable that the GFP reporter and the *aphA-3* cassette from pTM117 could readily be replaced with other reporter genes and resistance markers, respectively. For instance, *lacZ* or luciferase could likely replace the *gfpmut3* gene, and a chloramphenicol resistance gene has been shown to function in place of the *aphA-3* cassette (N. Salama personal communication). Thus, pTM117 can be further adapted to suit the needs of the individual researcher and project. Taken together, these data suggest that pTM117 could be a useful tool for the study of gene regulation in *H. pylori*.

There are two basic methods for achieving complementation within bacteria: complementation from a non-native locus within the chromosome or complementation by a gene carried on a plasmid. In *H. pylori*, disruption of *rdxA* has been described as a way

of complementing genes within the chromosome (11). While this method clearly takes advantage of the inherent properties of the *rdxA* locus, it nonetheless has a few potential confounders. First of all, metronidazole (Mtz) is a mutagen, and prolonged exposure to the drug likely results in second site mutations within the chromosome. Secondly, the rate of spontaneous Mtz resistance is often very high; it can range from $2.6x10^{-5}$ to $3.5x10^{-8}$ for various strains (24). Such a high rate could potentially require screening of hundreds of transformants to identify one that is Mtz resistant due to complementation within the *rdxA* locus. Therefore, while complementation within the *rdxA* locus can be beneficial, these potential confounders make it less than ideal. Thus, there is a real need for plasmid systems in *H. pylori* that can be used for complementation.

We have shown that pTM117 can be used to complement both iron-bound and *apo* Fur regulation in a *Δfur* mutant. Additionally, the pHel2 vector was able to partially complement iron-bound and *apo* Fur regulation in a Δ*fur* background although to a lesser extent than pTM117. This difference can be partially explained by the levels of *fur* expression obtained from each of the vectors. There is less *fur* expressed from pHel2 than from pTM117 as evidenced by the fainter bands in the pHel2 lanes of Fig. 3 (bottom panel) as compared to the WT lane and the pTM117 lanes. This is intriguing since pHel2 is predicted to have a higher copy number (four copies per cell (26) as compared to the one to two copies per cell of pTM117). Sequencing of the promoter regions and *fur* genes carried on both plasmids revealed that they were both identical to WT *fur*. Thus, the reason for this trend is currently unclear.

For pTM117 to be a truly useful tool for the *H. pylori* field, ideally it would need to be utilizable in *H. pylori* strains in addition to G27. To this end, we have

successfully transformed pTM117 or one of its derivatives into the gerbil colonizing stain, B128 (isolate 7.13, reference 19), the sequenced strain, HPAG1 (32), and the monkey colonizing strain, J166 (isolate 316-3.4, J. Solnick Lab Collection). In addition, pTM117 or its derivatives have successfully been moved into SS1 (29), 26695 (38), and J99 (1) (N. Salama and K. Ottemann personal communication). This fact combined with the results described herein indicates that pTM117 should be a useful option for complementation and transcriptional studies in a wide variety of strain backgrounds.

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References

1. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.

2. **Bauerfeind, P., R. M. Garner, and L. T. Mobley.** 1996. Allelic exchange mutagenesis of *nixA* in *Helicobacter pylori* results in reduced nickel transport and urease activity. Infect Immun **64:**2877-80.

3. **Bereswill, S., F. Lichte, T. Vey, F. Fassbinder, and M. Kist.** 1998. Cloning and characterization of the *fur* gene from *Helicobacter pylori*. FEMS Microbiol Lett **159:**193-200.

4. **Bereswill, S., R. Schonenberger, A. H. van Vliet, J. G. Kusters, and M. Kist.** 2005. Novel plasmids for gene expression analysis and for genetic manipulation in the gastric pathogen *Helicobacter pylori*. FEMS Immunol Med Microbiol **44:**157-62.

5. **Bijlsma, J. J., B. Waidner, A. H. Vliet, N. J. Hughes, S. Hag, S. Bereswill, D. J. Kelly, C. M. Vandenbroucke-Grauls, M. Kist, and J. G. Kusters.** 2002. The

Helicobacter pylori homologue of the ferric uptake regulator is involved in acid resistance. Infect Immun **70:**606-11.

6. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis **161:**626-33.

7. **Bury-Mone, S., J. M. Thiberge, M. Contreras, A. Maitournam, A. Labigne, and H. De Reuse.** 2004. Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. Mol Microbiol **53:**623-38.

8. **Chalker, A. F., H. W. Minehart, N. J. Hughes, K. K. Koretke, M. A. Lonetto, K. K. Brinkman, P. V. Warren, A. Lupas, M. J. Stanhope, J. R. Brown, and P. S. Hoffman.** 2001. Systematic identification of selective essential genes in *Helicobacter pylori* by genome prioritization and allelic replacement mutagenesis. J Bacteriol **183:**1259-68.

9. **Cormack, B. P., R. H. Valdivia, and S. Falkow.** 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173:**33-8.

10. **Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, and et al.** 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori*

associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A **90:**5791-5.

11. **Croxen, M. A., G. Sisson, R. Melano, and P. S. Hoffman.** 2006. The *Helicobacter pylori* chemotaxis receptor TlpB (HP0103) is required for pH taxis and for colonization of the gastric mucosa. J Bacteriol **188:**2656-65.

12. **de Vries, N., E. J. Kuipers, N. E. Kramer, A. H. van Vliet, J. J. Bijlsma, M. Kist, S. Bereswill, C. M. Vandenbroucke-Grauls, and J. G. Kusters.** 2001. Identification of environmental stress-regulated genes in *Helicobacter pylori* by a *lacZ* reporter gene fusion system. Helicobacter **6:**300-9.

13. **Delany, I., G. Spohn, A. B. Pacheco, R. Ieva, C. Alaimo, R. Rappuoli, and V. Scarlato.** 2002. Autoregulation of *Helicobacter pylori* Fur revealed by functional analysis of the iron-binding site. Mol Microbiol **46:**1107-22.

14. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2003. An anti-repression Fur operator upstream of the promoter is required for iron-mediated transcriptional autoregulation in *Helicobacter pylori*. Mol Microbiol **50:**1329-38.

15. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter*

pylori. Mol Microbiol **42:**1297-309.

16. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin Microbiol Rev **10:**720-41.

17. **Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. van Vliet, and G. Homuth.** 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. Microbiology **151:**533-46.

18. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J Bacteriol **174:**4212-7.

19. **Franco, A. T., D. A. Israel, M. K. Washington, U. Krishna, J. G. Fox, A. B. Rogers, A. S. Neish, L. Collier-Hyams, G. I. Perez-Perez, M. Hatakeyama, R. Whitehead, K. Gaus, D. P. O'Brien, J. Romero-Gallo, and R. M. Peek, Jr.** 2005. Activation of beta-catenin by carcinogenic *Helicobacter pylori*. Proc Natl Acad Sci U S A **102:**10646-51.

20. **Gancz, H., S. Censini, and D. S. Merrell.** 2006. Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen *Helicobacter pylori*. Infect Immun **74:**602-14.

21. **Goodwin, A., D. Kersulyte, G. Sisson, S. J. Veldhuyzen van Zanten, D. E. Berg, and P. S. Hoffman.** 1998. Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. Mol Microbiol **28:**383-93.

22. **Guo, B. P., and J. J. Mekalanos.** 2001. *Helicobacter pylori* mutagenesis by mariner in vitro transposition. FEMS Immunol Med Microbiol **30:**87-93.

23. **Guo, B. P., and J. J. Mekalanos.** 2002. Rapid genetic analysis of *Helicobacter pylori* gastric mucosal colonization in suckling mice. Proc Natl Acad Sci U S A **99:**8354-9.

24. **Haas, C. E., D. E. Nix, and J. J. Schentag.** 1990. In vitro selection of resistant *Helicobacter pylori*. Antimicrob Agents Chemother **34:**1637-41.

25. **Heuermann, D., and R. Haas.** 1995. Genetic organization of a small cryptic plasmid of *Helicobacter pylori*. Gene **165:**17-24.

26. **Heuermann, D., and R. Haas.** 1998. A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. Mol Gen Genet **257:**519-28.

27. **Josenhans, C., S. Friedrich, and S. Suerbaum.** 1998. Green fluorescent protein as a novel marker and reporter system in *Helicobacter* sp. FEMS Microbiol Lett **161:**263-73.

28. **Langford, M. L., J. Zabaleta, A. C. Ochoa, T. L. Testerman, and D. J. McGee.** 2006. In vitro and in vivo complementation of the *Helicobacter pylori* arginase mutant using an intergenic chromosomal site. Helicobacter **11:**477-93.

29. **Lee, A., J. O'Rourke, M. C. De Ungria, B. Robertson, G. Daskalopoulos, and M. F. Dixon.** 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology **112:**1386-97.

30. **Lee, W. K., Y. S. An, K. H. Kim, S. H. Kim, J. Y. Song, B. D. Ryu, Y. J. Choi, Y. H. Yoon, S. C. Baik, K. H. Rhee, and M. J. Cho.** 1997. Construction of a *Helicobacter pylori-Escherichia coli* shuttle vector for gene transfer in *Helicobacter pylori*. Appl Environ Microbiol **63:**4866-71.

31. **Merrell, D. S., L. J. Thompson, C. C. Kim, H. Mitchell, L. S. Tompkins, A. Lee, and S. Falkow.** 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. Infect Immun **71:**6510-25.

32. **Oh, J. D., H. Kling-Backhed, M. Giannakis, J. Xu, R. S. Fulton, L. A. Fulton, H. S. Cordum, C. Wang, G. Elliott, J. Edwards, E. R. Mardis, L. G.**

Engstrand, and J. I. Gordon. 2006. The complete genome sequence of a chronic atrophic gastritis *Helicobacter pylori* strain: evolution during disease progression. Proc Natl Acad Sci U S A **103:**9999-10004.

33. **Salama, N. R., G. Otto, L. Tompkins, and S. Falkow.** 2001. Vacuolating cytotoxin of *Helicobacter pylori* plays a role during colonization in a mouse model of infection. Infect Immun **69:**730-6.

34. **Salama, N. R., B. Shepherd, and S. Falkow.** 2004. Global transposon mutagenesis and essential gene analysis of *Helicobacter pylori*. J Bacteriol **186:**7926-35.

35. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harb Symp Quant Biol **43 Pt 1:**77-90.

36. **Tan, S., C. D. Fraley, M. Zhang, D. Dailidiene, A. Kornberg, and D. E. Berg.** 2005. Diverse phenotypes resulting from polyphosphate kinase gene (*ppk1*) inactivation in different strains of *Helicobacter pylori*. J Bacteriol **187:**7687-95.

37. **Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee, and S. Falkow.** 2003. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. Infect Immun **71:**2643-55.

38. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

39. **Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin.** 1985. In vivo transfer of genetic information between gram-positive and gram-negative bacteria. Embo J **4:**3583-7.

40. **Valdivia, R. H., and S. Falkow.** 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. Mol Microbiol **22:**367-78.

41. **van Amsterdam, K., A. H. van Vliet, J. G. Kusters, and A. van der Ende.** 2006. Of microbe and man: determinants of *Helicobacter pylori*-related diseases. FEMS Microbiol Rev **30:**131-56.

42. **van Vliet, A. H., E. J. Kuipers, J. Stoof, S. W. Poppelaars, and J. G. Kusters.** 2004. Acid-responsive gene induction of ammonia-producing enzymes in *Helicobacter pylori* is mediated via a metal-responsive repressor cascade. Infect Immun **72:**766-73.

43. **van Vliet, A. H., J. Stoof, S. W. Poppelaars, S. Bereswill, G. Homuth, M. Kist, E. J. Kuipers, and J. G. Kusters.** 2003. Differential regulation of amidase- and formamidase-mediated ammonia production by the *Helicobacter pylori* Fur repressor. J Biol Chem **278:**9052-7.

44. **Wang, G., T. J. Wilson, Q. Jiang, and D. E. Taylor.** 2001. Spontaneous mutations that confer antibiotic resistance in *Helicobacter pylori*. Antimicrob Agents Chemother **45:**727-33.

45. **Xiang, Z., S. Censini, P. F. Bayeli, J. L. Telford, N. Figura, R. Rappuoli, and A. Covacci.** 1995. Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. Infect Immun **63:**94-8.

Chapter Three

A Single Nucleotide Change Affects Fur-Dependent Regulation of sodB *in* H. pylori

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The work presented in this chapter is the sole work of B.M. Carpenter with the following exceptions: R.P. Gonzalez-Nieves aided in creating pDSM475, pDSM469, and DSM480, A.L. West and S.L. Michel helped analyze the competitive binding studies, and J.M. Whitmire generated the figures.

Abstract

Helicobacter pylori is a significant human pathogen that has adapted to survive the many stresses found within the gastric environment. Superoxide Dismutase (SodB) is an important factor that helps *H. pylori* combat oxidative stress. *sodB* was previously shown to be repressed by the Ferric Uptake Regulator (Fur) in the absence of iron (*apo*-Fur regulation) [1]. Herein, we show that *apo* regulation is not fully conserved among all strains of *H. pylori*. *apo*-Fur-dependent changes in *sodB* expression are not observed under iron-deplete conditions in *H. pylori* strains G27, HPAG1, or J99. However, Fur regulation of *pfr* and *amiE* occurs as expected. Comparative analysis of the Fur coding

sequence between G27 and 26695 revealed a single amino acid difference, which was not responsible for the altered *sodB* regulation. Comparison of the *sodB* promoters from G27 and 26695 also revealed a single nucleotide difference within the predicted Fur binding site. Alteration of this nucleotide in G27 to that of 26695 restored *apo*-Fur dependent *sodB* regulation, indicating that a single base difference is at least partially responsible for the difference in *sodB* regulation observed among these *H. pylori* strains. Fur binding studies revealed that alteration of this single nucleotide in G27 increased the affinity of Fur for the *sodB* promoter. Additionally, the single base change in G27 enabled the *sodB* promoter to bind to *apo*-Fur with affinities similar to the 26695 *sodB* promoter. Taken together these data indicate that this nucleotide residue is important for direct *apo*-Fur binding to the *sodB* promoter.

Introduction

Helicobacter pylori is an important human pathogen that infects over 50% of the world's population (18). While infection is predominantly asymptomatic, this bacterium is associated with development of gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma. Infection often occurs early in childhood and persists throughout a person's lifetime unless they are treated with specific antibiotics (4). Given its propensity for chronic colonization and the substantial number of infected individuals, *H. pylori* imposes a significant disease burden worldwide.

This microaerophilic, Gram negative bacterium is interesting in that it colonizes and survives within the gastric mucosa of the human stomach. *H. pylori* is well suited to life within this niche and has many factors that enable it to thrive there (18, 35). One

such factor, the *F*erric *u*ptake *r*egulator (Fur), functions as a transcriptional regulator that is involved in maintaining iron homeostasis (3). Iron is essential for bacterial survival and is a co-factor in a variety of proteins; however, iron is redox active and can promote oxidative damage making it imperative that intracellular iron levels are tightly controlled. One particularly deleterious reaction that free iron can promote is reaction with reactive oxygen species (ROS) to form highly reactive hydroxyl radicals via Fenton chemistry. Hydroxyl radicals cause DNA and cellular damage that eventually lead to cell death. Thus, cells must strive to maintain a balance between insufficient and excess iron. Fur is involved in preserving this fine balance in *H. pylori*, and consequently, it is not surprising that *fur* has been shown to be critical for colonization in both gerbil and murine models of infection (5, 23).

Fur is conserved in a wide variety of bacterial species and functions similarly in all of them by repressing gene expression under conditions of sufficient cellular iron. When Fur is bound to its iron (Fe^{2+}) co-factor, it binds to specific regions in iron-regulated promoters called Fur Boxes and blocks the binding of RNA polymerase. Genes regulated in this manner are often associated with iron acquisition and are repressed under iron replete conditions to prevent the harmful effects of iron overload. While *H. pylori* Fur has been found to repress a set of genes in its iron-bound state, it has also uniquely been found to repress an additional set of genes in the absence of the iron cofactor, i.e. when Fur is in its *apo* form. *apo*-Fur regulation involves repression of an iron storage gene and occurs under iron limited conditions (17).

apo-Fur regulation has not been described for other bacterial species, and given that Fur plays a role in global gene regulation in response to environmental stressors and

enhances the fitness of *H. pylori* as a pathogen, functional studies of Fur in *H. pylori* are of particular interest. One gene known to be repressed by *apo*-Fur in *H. pylori* that is not directly linked to iron metabolism, but is involved with the oxidative stress response, is *superoxide dismutase* (*sodB*) (20). SodB was first identified in *H. pylori* in 1993 and was shown to be iron co-factored like the *Escherichia coli* FeSod with 53.5% identity between the two proteins (32). However, unlike *E. coli* FeSod, which is localized within the cytosol of the bacterium, *H. pylori* SodB is associated with the cell surface (32). SodB is the only identified Sod in *H. pylori* and has been shown to be critical for survival *in vivo* (31). Also, *sodB* deficient mutants are more sensitive to O_2 as well as exhibit a higher rate of spontaneous mutation (31, 37). Interestingly, *H. pylori sodB* mutants have been shown to harbor more free iron within their cells than wild-type bacteria (37).

Globally, Sods are responsible for combating oxidative stress (both internal and external) by converting superoxide radicals into hydrogen peroxide and oxygen. Superoxide radicals are formed as a by-product of metabolism and, if left unchecked, can react with ferric iron (Fe^{3+}) to form hydrogen peroxide, which in turn feeds the Fenton Reaction (26) and is detrimental to the cell. Sods prevent the interaction of iron and superoxide radicals as well as block the formation of hydroxyl radicals from hydrogen peroxide (26). In this way, the role of Fur as the primary regulator of iron uptake and the role of SodB as the primary defense against superoxide radicals in *H. pylori* are linked. In keeping with this, *sodB* has been shown to be regulated by *apo*-Fur such that it is repressed under circumstances where iron is severely limited (20). This regulation appears to be direct since Electrophoretic Mobility Shift Assays showed that Fur specifically binds to the *sodB* promoter in the absence of iron (20). Herein we describe a

series of experiments that define a single polymorphic nucleotide within the *H. pylori sodB* promoter that is important for *apo*-Fur dependent regulation. Moreover, we show that alterations in this single base result in strain specific responses to iron limitation.

Materials and Methods

Bacterial strains and growth

Strains and plasmids used in this study are listed in Table 5, and primer sequences are listed in Table 6. Strains of *H. pylori* were maintained as frozen stocks at -80ºC in brain heart infusion broth (BD) supplemented with 10% fetal bovine serum (Gibco) and 20% glycerol (EMD Chemicals, Inc.). Bacterial strains were grown on horse blood agar (HBA) plates which contained 4% Columbia agar base (Neogen Corporation), 5% defibrinated horse blood (HemoStat Laboratories, Dixon, CA), 0.2% β-cyclodextrin (Sigma), 10µg/ml vancomycin (Amresco), 5µg/ml cefsulodin (Sigma), 2.5U/ml polymyxin B (Sigma), 5µg/ml trimethoprim (Sigma), and 8µg/ml amphotericin B (Amresco). Liquid cultures of *H. pylori* were grown in brucella broth (Neogen Corporation) supplemented with 10% fetal bovine serum and 10µg/ml vancomycin at 37ºC with shaking at 100 rpm. As noted in Table 5, where appropriate, cultures and plates were supplemented with 8µg/ml chloramphenicol (Cm) (EMD Chemicals, Inc.) and/or 25µg/ml kanamycin (Kan) (Gibco). In addition, where detailed in the Materials and Methods, some HBA plates contained 5% sucrose (Suc) (Sigma). Both liquid and plate cultures were grown under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) generated with an Anoxomat gas evacuation and replacement system (Spiral Biotech) in gas evacuation jars.

Table 5. Plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pTM117	Modified pHP666 to include *E. coli* origin and *rop* gene, *aphA-3* cassette (Kan^r), multiple cloning site, and a promoterless *gfpmut3* gene	(6)
pDSM236	pTM117 *sodB* promoter::*gfpmut3*fusion	This study
pDSM368	pTM117 *pfr* promoter::*gfpmut3*fusion	(6)
pKSF-II	pEK::*kan-sacB*	(7, 27)
pDSM386	pGEM-T Easy::Δ*fur*	This study
pDSM387	pGEM-T Easy::Δ*fur*::*kan-sacB*	This study
pDSM469	pGEM-T Easy::Δ*sodB*	This study
pDSM475	pGEM-T Easy::Δ*sodB*::*kan-sacB*	This study
pDSM481	pGEM-T Easy::*sodB* C-5A	This study
pDSM429	pGEM-T Easy::26695 *fur*	This study
pDSM430	pET21A::26695 *fur*	This study
pKD4	*kan* template plasmid	(11)
pKD46	Red recombinase expression plasmid	(11)
H. pylori strains		
G27	WT *H. pylori*	(10)
DSM300	G27 Δ*fur*::*cat,* Cm^r	(6)
26695	WT *H. pylori*	(19, 34)
DSM357	26695 Δ*fur*::*cat,* Cm^r	This study
DSM238	G27 (pDSM236), Kan^r	This study
DSM308	DSM300 (pDSM236), Kan^r Cm^r	This study
DSM369	G27 (pDSM368), Kan^r	(6)
DSM370	DSM300 (pDSM368), Kan^r Cm^r	(6)
DSM391	G27 Δ*fur*::*kan-sacB*, Kan^r Suc^s	This study
DSM403	G27, *fur* 26695, Suc^r Kan^s	This study
DSM480	G27 Δ*sodB*::*kan-sacB*, Kan^r Suc^s	This study
DSM491	G27 *sodB* C-5A, Suc^r Kan^s	This study
J99	WT *H. pylori*	(1)
HPAG1	WT *H. pylori*	(28)
E. coli strains		
DSM328	K12 (pKD46), Amp^r, Temp^s	(11)
DSM355	K12 Δ*fur*, Kan^r	This study

DSM326	BL21 DE3 Rosetta/pLysS, Cm^r	This study
DSM365	BL21 DE3 Rosetta/pLysS Δ*fur*, Kan^r, Cm^r	This study
DSM431	BL21Δ*fur* (pDSM430) Amp^r, Cm^r, Kan^r	This study

Table 6. Primers used in this study

Primer[b]	Sequence (5'-3')[a]	Reference
sodB promoter primers		
sodB-F1 (SacII)	CCGCGGCGCCATTGACCAATTTCAG	This study
sodB-R1 (BamHI)	GGATCCGCAACTCTCGTAATGTAAAC	This study
Screening and Sequencing primers		
gfp-1	AAGTCGTGCTGCTTCATGTG	(6)
aphA3-2	CGGTGATATTCTCATTTTAGCC	(6)
sacBSCN-F2	CGAATCGAATTCAGGAAC	This study
HpKanSacSCN-R	GGGAAGTTCTATGCTTATGG	This study
HpsodBSCN-R	GCTCGCTTCTTTAAACTCAACC	This study
Cloning primers		
FurCF (XbaI)	TCTAGAAAGGCTCACTCTACCCTATT	(6)
HpUKanSacR (XhoI, SmaI)	CTCTTGGCATTCTTTACACCACACCCCGGG**AGG**CTCGAGGCTGATATCTTCCTTATCCG	This study
HpDKanSacF (XhoI, SmaI)	CGGATAAGGAAGATATCAGCCTCGAG**CCT**CCCGGGGTGTGGTGTAAAGAATGCCAAGAG	This study
HpDKanSacR	CGCAGCGATAAAGGCGTGGTG	This study
FurCR (SalI)	GTCGACAAGACTTTCACCTGGAAACGC	(6)
USod-F	GCTTTATCGCCCACTTTCAAG	This study
USod-R	CCACAATAGCCGTAACGCTTACCCGGG**AGG**CTCGAGCATGTTTTCTCCTTGTGATTAG	This study
DSod-F	CTAATCACAAGGAGAAAACATGCTCGAG**CCT**CCCGGGTAAGCGTTACGGCTATTGTGG	This study
DSod-R	GGCATGGAATTGTCAATCC	This study
SodBMt-R	GATAGCCTTATTGTAATC	This study
SodBMt-F	GATTACAATAAGGCTATC	This study

HP_Fur_expression F2 (NdeI)	CATATGAAAAGATTAGAAACTTTGGAATCCATTTT	This study
HP_Fur_expression R2 (Xho1)	CTCGAGTTATTAACATTCACTCTCTTGG	This study
Red_EC_Fur_F	GAGCTGTAACTCTCGCTTTTCTTATTTCCCTTGCATGTGTAGGCTGGAGCTGCTTC	This study
Red_EC_Fur_R	TCATGTCTACGCCGTATTAATAGATAATGCCAATCACCATATGAATATCCTCCTTAGTTC	This study
RPA primers		
amiE-RPA-F	GGTTTGCCTGGGTTGGAT	(23)
amiE-RPA-R	GATTTTGCGGTATTTTG	(23)
pfr-RPA-F	GCGGCTGAAGAATACGAG	(6)
pfr-RPA-R	CTGATCAGCCAAATACAA	(6)
sodB-RPA-F	AAGCCCTGTAGCGTTTGATT	This study
sodB-RPA-R	CCCAATTCCAACCAGAGCCA	This study
fur RPA F	GAGCGCTTGAGGATGTCTATC	(6)
fur RPA R	GTGATCATGGTGTTCTTTAGC	(6)
EMSA primers		
G27 sodB EMSA-F	CTACAAAATTTGCATAACG	This study
26695 sodB EMSA-F	CCACAAAATTTGCATAAAG	This study
sodB EMSA-R	GCAACTCTCGTAATGTAAAC	This study
rpoB EMSA-F	CCAAAGAGGGTAAAGAGAGCG	This study
rpoB EMSA-R	CCTCTCCATCGCTTCTCTAAC	This study

[a]Restriction endonuclease sites are underlined, and linker bases are in bold type.

[b]Important restriction sites are included in parentheses

H. pylori strains used in this study are all derivatives of G27 (10) and 26695 (19, 34), with the exception of WT *H. pylori* J99 (1) and HPAG1 (28). A *fur* (HP1027) mutant of G27, DSM300, was utilized in this work and contains a deletion insertion of the *fur* coding sequence with the *cat* gene from *Campylobacter coli* conferring Cm resistance as previously described (6). This ΔHP1027::*cat* construct was also naturally transformed into 26695 to create an analogous *fur* mutation in this strain background and is called DSM357. Exponential phase cultures were grown for 20hrs, and stationary phase cultures were grown for 44hrs.

Creation of the sodB *promoter fusion plasmid*

A transcriptional fusion of the *sodB* (HP0389) promoter to the promoterless *gfpmut3* on the transcriptional reporter plasmid, pTM117, was constructed as previously described (6). Briefly, the *sodB* promoter of WT G27 was PCR amplified using sodB-F1 and sodB-R1 primers, which incorporate SacII and BamHI restriction sites, respectively. The resulting PCR fragment was subcloned into pGEM-T Easy (Promega) and digested with SacII (New England Biolabs) and BamHI (Invitrogen). The resultant promoter fragment was then ligated into the appropriately digested pTM117 vector to create pDSM236. The fusion was confirmed by PCR amplification with sodB-F1 and gfp-1 (6) primers and by sequencing with the aphA3-2 primer (6). pDSM236 was naturally transformed into WT G27 and DSM300, and transformants were selected on HBA plates containing 25μg/ml Kan and 25μg/ml Kan plus 8μg/ml Cm, respectively. The WT strain bearing pDSM236 was designated DSM238, and DSM300 bearing pDSM236 was designated DSM308.

GFP reporter assays

The ability of the *sodB* transcriptional fusion to drive the expression of GFP was assessed using flow cytometry as described previously (6). Briefly, DSM238 was grown overnight in liquid culture with and without the iron chelator, 2,2'-dipyridyl (dpp) (Sigma) at a final concentration of 60μM, and DSM308 was grown overnight in the absence of chelator. As a comparison, the previously characterized strains, DSM369 and DSM370, which bear *pfr* (*nonheme iron-containing ferritin*) transcriptional fusion plasmids in WT and Δ*fur* G27, respectively, were grown in the same manner (6). Following overnight growth, 1.5ml of each culture were pelleted and resuspended in 1ml of sterile 1x phosphate-buffered saline. Bacterial clumps and culture debris were subsequently removed by passing the resuspended culture through a 1.2-μm Acrodisc PSF syringe filter (Pall). Flow cytometry analysis was performed using a Beckman Coulter Epics XL-MCL flow cytometer with a laser setting of 750V for the *pfr* fusion construct and 900V for the sodB fusion construct. 100,000 events were collected for each assay. WinList 3D, version 6.0 (Verity Software House) was used to analyze the flow cytometry data.

Creation of the "Fur swap" Strain

To exchange the *fur* coding sequence, we first created a G27 strain containing the counter-selectable *kan-sacB* cassette previously described by Copass, et al (7). This cassette contains the *sacB* gene from *Bacillus subtilis,* which confers Suc sensitivity and is expressed under the control of the *flaA* promoter of *H. pylori*, and the *aphA3* gene from

Campylobacter coli, which confers Kan resistance. A 340bp region upstream of the G27 *fur* coding sequence was PCR amplified using primers FurCF1 (6) and HpUKanSacR, and a 339bp region downstream of the *fur* coding sequence was PCR amplified using primers HpDKanSacF and HpDKanSacR. HpUKanSacR and HpDKanSacF were designed to incorporate XhoI and SmaI restriction endonuclease sites. Each of these products were purified and mixed in a Splicing by Overlap Extension (SOE) PCR reaction using the FurCF1 and HpDkanSacR primers. The resultant 679bp product was subcloned into pGEM-T Easy creating pDSM386. The *kan-sacB* cassette was liberated from pKSF-II (7, 27) by sequential double digestion with XhoI (New England Biolabs) and SmaI (New England Biolabs), and this fragment was ligated to the appropriately digested pDSM386 to create pDSM387. This plasmid was naturally transformed into WT G27, and transformants were selected on HBA plates containing Kan. Double crossover homologous recombination of pDSM387 with the WT chromosome results in the complete deletion of the *fur* (HP1027) coding sequence and replacement with the upstream *fur-kan-sacB*-downstream *fur* product. The resulting transformants were patched on 5% Suc HBA plates to ensure Suc sensitivity, and proper integration into the chromosome was confirmed by PCR with sacBSCN-F2 and HpKanSacSCN-R primers, which lie within the *sacB* gene and downstream of fur, respectively. One such transformant was named DSM391.

To create the "Fur swap" strain, a 923bp product of the *H. pylori* 26695 genome was amplified using the FurCF and FurCR primers. This product, which includes the *fur* coding sequence and a portion of the upstream and downstream regions, was purified and naturally transformed into DSM391. Transformants were selected on 5% Suc HBA

plates and patched onto Kan HBA plates to ensure Kan sensitivity. Double crossover homologous recombination resulted in the replacement of the *kan-sacB* cassette with the *fur* coding sequence of 26695, and this strain was named DSM403. Proper integration was confirmed by PCR with the FurCF and FurCR primers and by sequencing with the FurCR primer. DSM403 expresses 26695 *fur* from the native *fur* locus in a G27 strain background.

Creation of a "-5bp swap" mutation in the sodB *promoter*

The *sodB* promoter from G27 was sequenced using primers USod-F and DSod-R and compared to the known sequence of the *sodB* promoter from 26695 (34). This comparison revealed a single base pair (bp) difference within the predicted Fur Box (20) at the -5 position relative to the start of transcription. The "-5bp swap" mutation within the *sodB* promoter of G27 was created using SOE PCR and the *kan-sacB* cassette from pKSF-II. A 297bp region upstream and a 329bp region downstream of *sodB* were PCR amplified from G27 using primer pairs USod-F and USod-R and DSod-F and DSod-R, respectively. USod-R and DSod-F contain XhoI and SmaI restriction endonuclease sites to allow for the directional cloning of the *kan-sacB* fragment. The upstream and downstream products were purified and mixed in a SOE PCR reaction with the USod-F and DSod-R primers. The resultant 626bp SOE PCR product was subcloned into pGEM-T Easy to create pDSM469. pDSM469 and pKSF-II were each sequentially double digested with XhoI and SmaI, and the resulting fragments were ligated to create pDSM475. pDSM475 was naturally transformed into WT G27, and transformants were selected on Kan and then patched to verify sucrose sensitivity. Double crossover

homologous recombination of pDSM475 into the G27 chromosome results in the deletion of the *sodB* gene and replacement with the *kan-sacB* cassette. The resulting Kan resistant, sucrose sensitive strain, DSM480, was confirmed by PCR with sacBSCN-F2 and HpsodBSCN-R primers, the latter of which lies downstream of *sodB*.

The -5bp in the G27 *sodB* promoter was mutated from a C to an A using SOE PCR. First, primers USod-F and SodBMt-R were used to PCR amplify upstream of the *sodB* promoter through to the -5bp and incorporate the C-5A mutation. Second, primers DSod-R and SodBMt-F were used to PCR amplify from the -5bp through to downstream of the sodB gene and to incorporate the C-5A mutation. These products were purified and combined in SOE PCR reaction using the USod-F and DSod-R primers. The resulting SOE PCR product was sublconed into pGEM-T Easy. The subcloned *sodB* -5bp promoter mutation construct was designated pDSM481 and was confirmed by sequencing with the USod-F and DSod-R primers.

pDSM481 was naturally transformed into DSM480 to integrate the *sodB* -5bp promoter mutation into the chromosome in place of the *kan-sacB* cassette. Transformants were selected as detailed above for the creation of DSM403. The resulting Suc resistant, Kan sensitive strain was named DSM491. Proper recombination was confirmed by PCR with the USod-F and DSod-R primers (yielding a 1,262bp fragment) and by sequencing with both of those primers. DSM491 expresses *sodB* with the C-5A mutation from its native locus within the G27 chromosome.

RNase protection assays (RPAs)

RPAs were utilized to characterize *apo*-Fur regulation of *sodB* in various strains of *H. pylori*. Two normal (iron replete) media cultures were started for each strain, one for exponential and one for stationary growth phase. Following overnight growth, one half of each exponential phase culture was removed for RNA isolation. To the remaining half of the iron-replete exponential phase cultures, 200μM dpp (final concentration) was added to create an iron-depleted shock condition. Those cultures were grown for an additional hour prior to RNA isolation. The iron-replete stationary phase cultures were grown for an additional night, and on the following morning one half of the culture was removed for RNA isolation while the other was exposed to 200μM dpp for an additional hour before RNA isolation. In addition, one culture for each strain was grown in iron limited media (60μM dpp). After overnight growth, one-half of each culture was removed for RNA isolation in exponential phase. The remaining half of the iron-limited growth culture was allowed to grow overnight and was harvested the following morning for the stationary phase, iron-limited growth RNA samples. RNA was extracted as described previously (33). RNase Protection Assays (RPAs) were performed as previously described (6) with 1.5μg of RNA using *sodB*, *pfr*, *amiE*, and/or *fur* riboprobes that were generated using the primer pairs listed in Table 6. In brief, riboprobes were generated with 50μCi [^{32}P]UTP (Perkin-Elmer) and a Maxiscript kit (Applied Biosystems). The RPA III kit (Applied Biosystems) was used for the RPA reactions that were resolved on 5% acrylamide-1X Tris-borate-EDTA-8M urea denaturing gels. The gels were exposed to phosphor screens, and the phosphor screens were scanned using a FLA-5100 multifunctional scanner (Fujifilm). Analyses and quantitation of the RPAs

were performed using the Multi-Gauge software (version 3.0, Fujifilm). In all cases, three to four biological repeats of each experiment were performed.

H. pylori *Fur Expression and Purification*

H. pylori 26695 Fur coding sequence was amplified using primers HP_Fur_expression F2 (NdeI) and HP_Fur_expression R2 (XhoI), and the PCR product was cloned into the pGEM-T easy vector (Promega) to create plasmid pDSM429. pDSM430 was created by proper digestion of pET21A (Novagen) and pDSM429 with NdeI and XhoI and ligation of the gel purified fragments. The Fur coding region in pDSM430 was sequenced to verify the construct. To prevent cross contamination of *H. pylori* recombinant Fur with *E. coli* endogenous Fur, an *E. coli* BL21 Rosetta *Δfur* strain was constructed using the Wanner method (11). Briefly, the Kan resistance cassette was amplified from pKD4 (11) with primers Red_EC_Fur_F and Red_EC_Fur_R. This PCR product was introduced into arabinose induced *E. coli* K-12 carrying the pKD46 plasmid (11) to create DSM355. DSM365 was created by transduction of DSM326 with P1L4 grown on DSM355. Endogenous *E. coli* Fur deletion was verified by PCR. pDSM430 was introduced into DSM365 to create DSM431, which was used for rFur induction. DSM431 was grown to mid log in Luria-Bertani (EMD Chemicals) medium and then induced with 0.5mM IPTG (isopropyl-D-thiogalactopyranoside) (Sigma) at 30°C for 3 h. The cells were disrupted using French press (Amicon) and crude extracts were prepared from the IPTG-induced cells by centrifugation (5,000 rpm for 30 minutes). Protein purification was performed by fast-protein liquid chromatography; the cytoplasmic protein was first passed through a HiTrap SP column for ion-exchange-based purification

with a salt gradient of 25mM to 500mM NaCl (obtained by using buffer A [50mM sodium phosphate, 25mM NaCl, pH 8.0] and buffer B [25mM sodium phosphate, 500mM NaCl, pH 8.0]). Peak fractions containing Fur protein from the ion-exchange procedure were collected and further purified based on size exclusion by using a Sephacryl-200 column (buffer C [50mM sodium phosphate, 200mM NaCl, pH 8.0]). rFur was partially concentrated using an Amicon Ultra Centrifugal Filter Device (Millipore) to remove a portion of buffer C. Then an equal volume of EMSA binding buffer (BB) was added to the partially concentrated rFur with an additional 50% glycerol. rFur was further concentrated before being quantitated and stored at -20ºC. The final concentration of the rFur stock was 2mg/mL.

Electrophoretic Mobility Shift Assays (EMSAs)

A 120bp region of the *sodB* promoter (encompassing the Fur-box) (20) was PCR amplified using the following template and primer pairs: WT G27 and DSM491 ("-5bp swap") with G27 sodB EMSA-F and sodB EMSA-R and WT 26695 with 26695 sodB EMSA-F and sodB EMSA-R. To serve as a negative control in the EMSA studies, a 142bp region of the *rpoB* promoter was amplified from WT G27 using the rpoB EMSA-F and rpoB EMSA-R primer pair. Each PCR product was acrylamide gel purified and resuspended in 1x Tris-EDTA (TE) buffer. 150ng of each promoter region was end labeled with [^{32}P] ATP (Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs) as previously described (23). The unincorporated nucleotide was removed using the MinElute Reaction Clean-up kit (Qiagen), and labeled promoter fragments were eluted twice with 10µL EB, and 50µL of *apo*-BB was added to the eluted product.

EMSAs were performed under *apo* (iron-free) conditions as previously described for WT 26695 *sodB* (20). Briefly, 1ng of labeled *sodB* or *rpoB* promoter was mixed with 5µL of the following dilutions of the Fur stock: 1:1,875, 1:3,125, 1:15,625, and 1:78,125 and combined with 10µL of 2x *apo*-BB (24% glycerol, 40mM Tris, pH 8.0, 150mM KCl, 2mM DTT, 600µg/mL bovine serum albumin, 200µM EDTA, and 0.1mg/mL sheared salmon sperm DNA). In addition, a no protein control reaction and a 100ng cold (unlabeled) DNA competition reaction were performed. The cold competition reaction was performed with the highest concentration of Fur (1:1,875). All reactions were allowed to incubate at 37ºC for 30min. After the incubation, the reactions were separated on a 5% polyacrylamide gel (5% 19:1 acrylamide, 1x Tris Glycine EDTA (TGE) buffer, 2.5% glycerol) for 3 hours at 70V in 1xTGE buffer. The gels were then exposed to phosphor screens and scanned on a Storm 860 scanner (GE Healthcare). Analysis was performed using ImageQuant version 5.2 software (Molecular Dynamics).

Competition EMSA Studies

Competition studies were performed in a manner analogous to the EMSAs. Each labeled *sodB* promoter fragment was combined with the 1:1,875 dilution of rFur, *apo*-BB, and either 5ng, 10ng, or 25ng of cold (unlabeled) *sodB* promoter from each of the three respective strains. A no competitor control was included for each labeled *sodB* promoter fragment. In this manner, each labeled *sodB* fragment (WT G27, "-5bp swap," and WT 26695) competed for binding to Fur with its own unlabeled *sodB* fragment as well as to that of the other two strains. The incubations, electrophoresis, and analysis were performed as described for the EMSAs. Binding competition occurs as follows:

$PD_{P32} + D \longleftrightarrow PD + D_{P32}$, where P = Fur, D_{P32} = labeled DNA, and D = cold competitor. Thus, if the competitor promoter fragment (D) can bind to Fur (P) with a higher affinity than the labeled promoter (D_{P32}), then an increase in the amount of unbound, labeled promoter (D_{P32}) would be seen. The percent of unbound, labeled *sodB* promoter was quantitated for each competition EMSA using densitometry as a means of comparing the relative affinity of each promoter fragment for Fur.

Statistical Analysis

Two-tailed Student's *t*-tests were performed using Microsoft Office Excel 2003.

Nucleotide sequence accession number

The nucleotide sequence of the *sodB* promoter is available from GenBank under accession number EU888136. The G27 *fur* sequence was previously reported (6) and is available as GenBank accession number EF537051.

Results

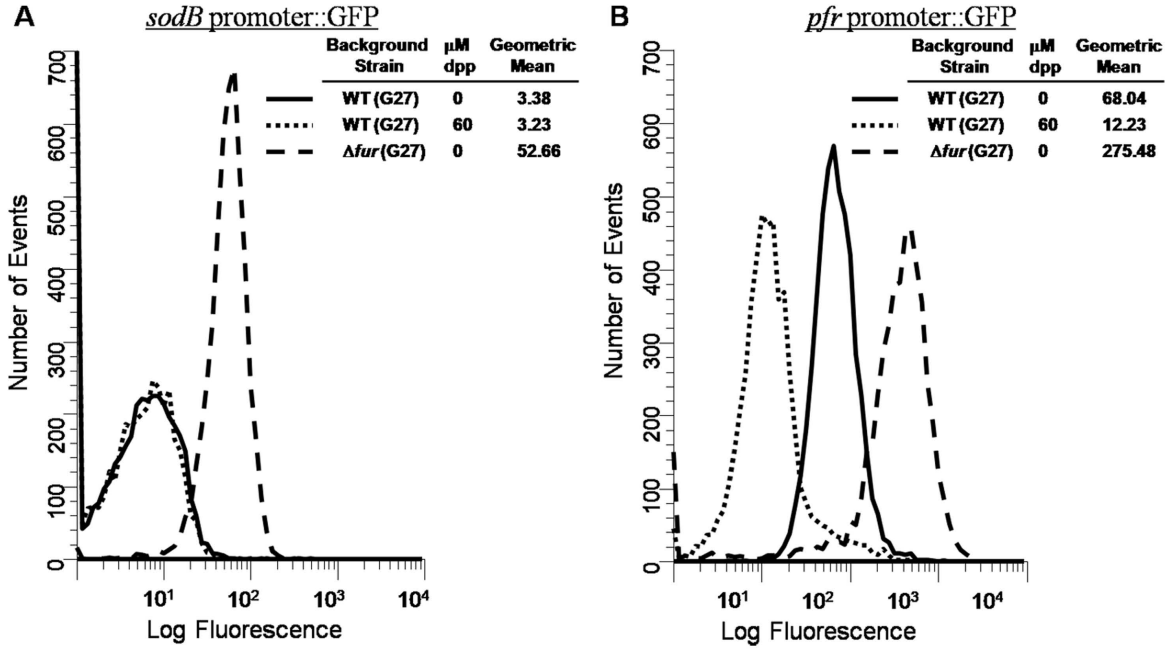
apo-*Fur Regulation in* H. pylori

In order to study *apo*-Fur dependent regulation in *H. pylori*, the *sodB* and *pfr* promoters from strain G27 were fused to the promoterless *gpfmut3* gene in pTM117. Currently, these promoters represent the only known targets of *apo*-Fur (17, 20). Given this *apo*-regulation and since promoter activity can be measured by changes in fluorescence with our system, we expected to see a decrease in GFP fluorescence under iron limited conditions for both promoter fusions. However, as shown in Fig.7A, the

Figure 7. Flow cytometry analysis of sodB *and* pfr *GFP reporters.*

Strains bearing *sodB::gfpmut3* or *pfr::gfpmut3* promoter fusions were grown overnight in either iron replete or iron depleted media. Changes in fluorescence were analyzed as described in the Materials and Methods. Results for the *sodB* promoter fusions are displayed in Panel 1A, and results for the *pfr* promoter fusions are displayed in Panel 1B. For both A and B, solid lines indicate the plasmid in WT *H. pylori* G27 grown in iron replete conditions, dotted lines indicate the plasmid in WT bacteria grown in iron deplete conditions, and dashed lines indicate the plasmid in Δ*fur* bacteria grown in iron replete conditions. Fluorescence is measured in relative units, and the data are representative of multiple independent flow analyses.

Figure 7. Flow cytometry analysis of sodB *and* pfr *GFP reporters.*



addition of iron chelator resulted in no change in the level of *sodB* expression. This is in contrast to *pfr*, where iron depletion resulted in strong repression of *pfr* expression (Fig. 7B). Both *sodB* and *pfr* were upregulated in a *fur* mutant (Fig. 7A and 7B) suggesting that both genes are repressed by Fur. However, the lack of responsiveness to iron chelation suggested that *sodB apo*-regulation is not as expected in G27.

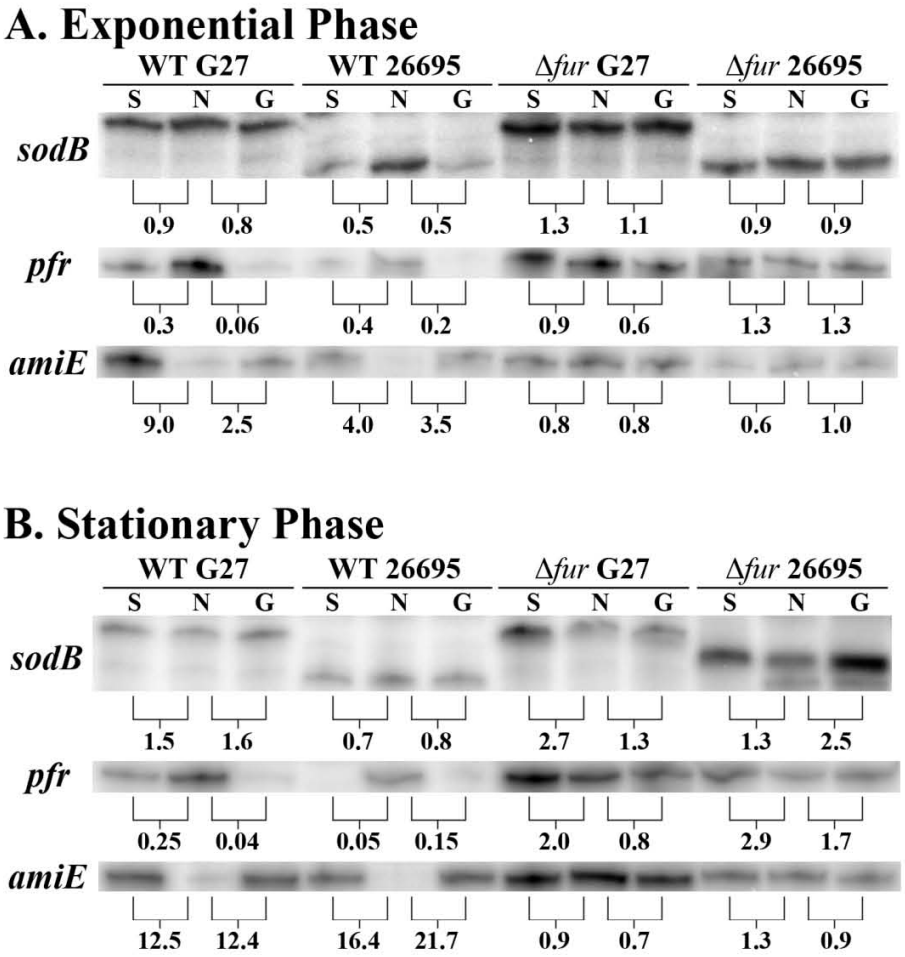
Since *apo*-Fur has been shown to have a lower affinity for the *sodB* promoter than the *pfr* promoter, and since the *gfpmut3* allele encodes a long-lived GFP variant (8), we reasoned that we might not be able to detect small changes in GFP expression under the control of the *sodB* promoter under iron limited conditions. Therefore, we performed RPAs to further investigate the discrepancy between our results and results previously reported for *sodB* regulation in strain 26695 (20). Additionally, we considered the fact that strain-specific differences might be responsible for the discrepancy. Therefore, RPAs using a *sodB* riboprobe were performed on RNA isolated from WT and Δ*fur* derivatives from both G27 and 26695. *pfr* and *amiE* (*aliphatic amidase*) riboprobes were also used as control *apo*-Fur and iron-bound Fur regulated target genes, respectively. Fig. 8A shows results for all three riboprobes using RNA isolated from exponential phase cultures. Again, we observed that for G27 the level of *sodB* expression did not change under iron-limited growth conditions (G) or under a harsher iron-depletion shock condition (S) that was added to ensure robust chelation as compared to normal (N) iron replete conditions.

Examination of *sodB* expression in 26695 revealed a smaller protected fragment than originally expected. However, sequence analysis revealed that the smaller fragment is due to a small region of mismatch between the *sodB* mRNA sequence in 26695 and the

Figure 8. Direct Comparison of sodB *Regulation in* H. pylori *Strains G27 and 26695.*

WT and Δ*fur* strains of G27 and 26695 were grown to exponential (A) and stationary (B) phase in iron replete and iron-limited (growth) media (60μM dpp). After growth overnight, one-half of the exponential phase, iron-replete culture was removed for RNA isolation. 200μM dpp (final concentration) was added to create an iron-depletion shock condition to the remaining half of the iron-replete cultures, and those cultures were grown for an additional hour prior to RNA isolation. The same procedure was applied the following day to the iron replete, stationary phase culture. After overnight growth, one-half of the iron-limited growth culture was removed for RNA isolation in exponential phase while the remaining half was allowed to grow into stationary phase, and RNA was isolated the following day. RNase Protection Assays (RPAs) were performed on RNA isolated from these strains using *sodB*, *pfr*, and *amiE* riboprobes. Data for Exponential phase cultures are shown in Panel A, and data for Stationary phase cultures are shown in Panel B. Fold-changes are indicated below each pair and were calculated by comparing either the relative amount of protected riboprobe in the iron-depletion shock environment (S) or the relative amount of protected riboprobe in the iron-limited growth environment (G) to the iron replete lane (N). These data are representative of multiple independent experiments.

Figure 8. Direct Comparison of sodB *Regulation in* H. pylori *Strains G27 and 26695.*



G27 template DNA used to generate the riboprobe. This mismatch causes a bubble of single stranded RNA to form and thus is subjected to RNase cleavage in the region of mismatch (data not shown). For WT 26695, a 2-fold decrease in *sodB* expression was achieved under both iron-limited growth and iron-depletion shock conditions, which agrees with the previous report (20). This change is Fur-dependent as there is no change in *sodB* expression under either iron depletion condition in the absence of *fur*.

Since it has been shown that growth phase strongly affects gene expression in *H. pylori* (33), we performed similar experiments on RNA harvested from stationary phase cultures. As shown in Fig. 8B, we obtained identical results with the exception that the fold decrease seen in *sodB* expression was less pronounced in 26695 in this growth phase. Again, there was no decrease in *sodB* expression in G27, indicating that growth phase is not responsible for the differences in our results. Moreover, the difference in *sodB* regulation between the two strains is not the result of a generalized difference in *apo*-Fur regulation between G27 and 26695 since the appropriate decrease in *pfr* expression (17) was observed in both strains under iron-limited growth and iron-depletion shock conditions (Fig 2A and 2B). Furthermore, iron-bound Fur regulation of *amiE* was as expected (36) for both G27 and 26695; *amiE* expression was increased under both iron limited conditions (Fig. 8A and 8B). Taken in total, these data indicate that *apo*-Fur regulation of *sodB* is altered in G27 as compared to 26695.

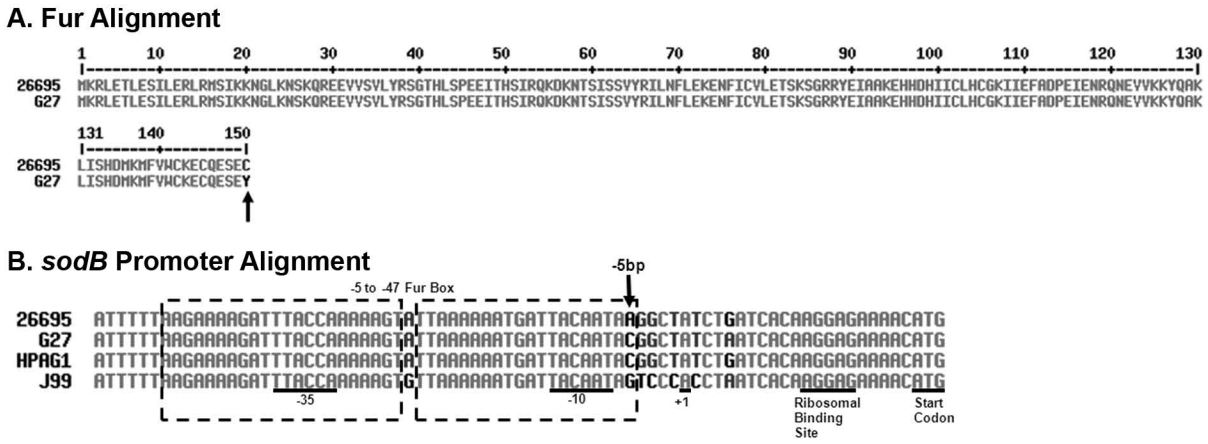
Analysis of the role an amino acid (AA) difference in Fur plays in sodB *regulation.*

Given the difference in *sodB* regulation between the two strains, we reasoned that either a difference in Fur or a difference in *sodB* between the two strains was likely to be

Figure 9. Alignments of Fur and of the sodB *promoters*.

Panel A contains the alignment of the predicted Fur amino acid sequences of G27 and 26695. As indicated by an arrow, amino acid 150 is different between the two strains. Panel B contains the *sodB* promoter alignment from G27, 26695, J99, and HPAG1 with essential promoter elements indicated. The predicted Fur Box ranges from bases -5 to -47 and is indicated by the dashed box (20). The -5bp difference between the strains is indicated with an arrow in Panel B. Alignments for both panels were constructed using MultAlin software (9).

Figure 9. Alignments of Fur and of the sodB *promoters.*



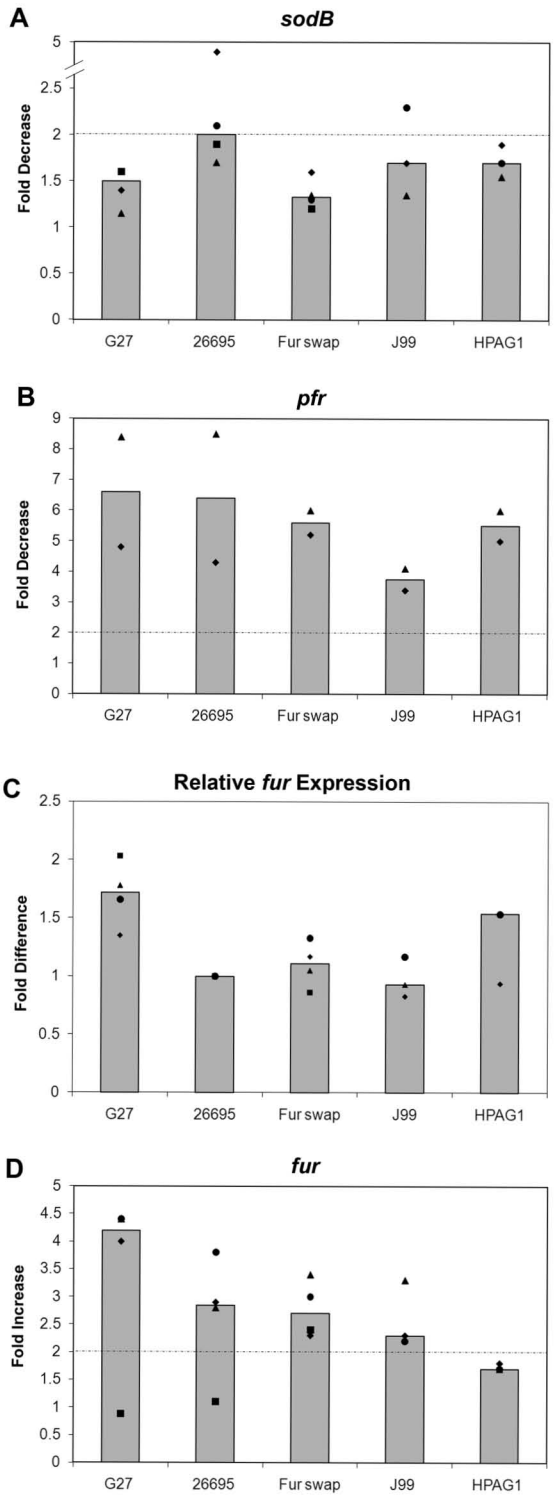
responsible for the change. We therefore aligned the predicted Fur amino acid sequence from G27 and 26695 to determine if there were any obvious differences between the two strains that might account for the differences in *sodB* regulation. As shown in Fig. 9A, the last AA was found to differ between the strains. In G27 AA 150 is a Tyr while in 26695 it is a Cys. To determine if this AA difference had any role in Fur-dependent regulation of *sodB*, a "Fur swap" strain was created, which completely replaced the G27 *fur* coding sequence with the coding sequence from 26695. RPAs were then conducted on RNA harvested from WT G27, WT 26695, and the "Fur swap" strain. Results are shown in Fig. 10. In order to show the reproducibility of the data, RPA data are represented in a graphical format. In this manner the fold change for each strain and biological repeat is displayed as a point on the graph. Additionally, the median fold change is depicted as a bar to allow for easy comparison between the strains. Because the decrease in *sodB* expression in 26695 is most pronounced in exponential phase, only results of RPAs performed using exponential phase RNA are shown. Expressing 26695 Fur in G27 (the "Fur swap" strain) did not restore *apo*-Fur *sodB* regulation in G27 under either iron-limited growth or iron-depletion shock conditions (Fig. 10A and data not shown). However, *apo*-Fur regulation of *pfr* was as expected in all three strains (Fig. 10B and data not shown) (17). Because the trends of the growth data for both the *sodB* and *pfr* RPA data were similar to the shock, the growth data has not been shown.

While the AA difference in Fur was apparently not responsible for the difference in *sodB* regulation, we wondered if the levels of *fur* expression were similar between the different strains. To test this, RPAs were performed on RNA isolated from all three strains using a *fur* riboprobe. The basal level of *fur* expression in each strain was then

Figure 10. Strain-specific differences in sodB *regulation.*

Various *H. pylori* strains were grown to exponential phase as described in the Materials and Methods, and RNA was isolated from iron replete and iron-depleted shock conditions. RPAs were performed using *sodB*, *pfr*, and *fur* riboprobes and results are displayed in Panels A, B, and D, respectively. Basal levels of *fur* expression relative to the level of expression in 26695 are depicted in Panel C. Fold decrease in expression for *sodB* and *pfr*, fold increase for *fur*, and relative levels of basal *fur* expression are plotted as single points for each strain with squares, diamonds, triangles, and circles. Each shape represents a biologically independent set of RNA. Median fold change is represented as a bar for each strain. The dotted-dashed line represents the 2-fold significance cut-off in Panels A, B, and D. In Panel A only, the triangles represent the average of two technical repeats on that independent set of RNA.

Figure 10. Strain-specific differences in sodB *regulation.*



compared to that of WT 26695 as shown in Fig. 10C. While the level of *fur* expression in the G27 strain was slightly higher than in 26695, no substantial differences in *fur* expression were found between the strains.

As Fur has been shown to be autoregulatory, repressing its own expression in the presence of iron (15-16), we also compared Fur autoregulation between G27, 26695, and the "Fur swap" strain. *fur* RPAs were performed on RNA isolated from each strain, and an increase in *fur* expression was seen for G27, 26695, and the "Fur swap" strain under iron-depletion shock conditions while little to no increase was seen under iron-limited growth conditions (Fig. 10D and data not shown). This data shows that Fur autoregulation is consistent in each strain and further supports the notion that the AA difference in Fur is not responsible for the difference in *sodB* regulation between G27 and 26695.

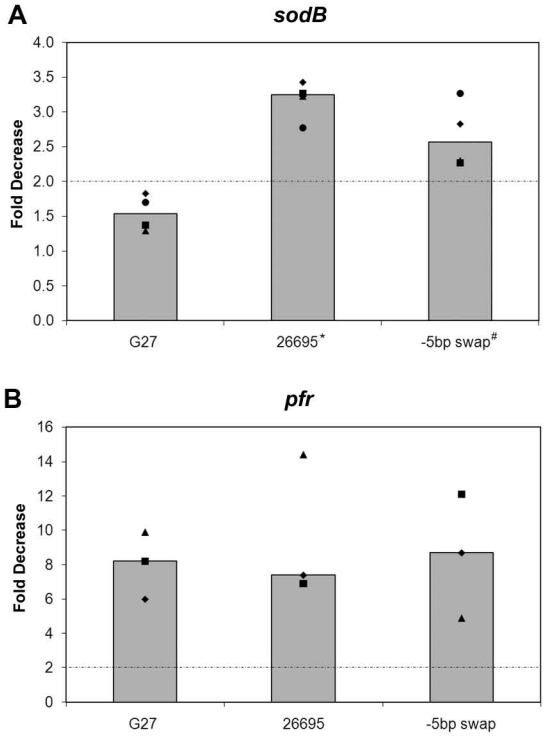
RPA determination of the role the -5bp of the sodB *promoter plays in* sodB *regulation.*

Since the difference in *sodB* regulation between G27 and 26695 appeared not to be related to the difference in the Fur coding sequence, we next considered that there might be differences in the *sodB* promoter between the strains that could account for the discrepancy in regulation. Therefore, we sequenced the *sodB* promoter from G27 and compared it to the known *sodB* promoter sequence from 26695 (34). As shown in Fig. 9B, a single base change was evident in the Fur Box. Previous DNA Footprint analysis showed that Fur protects a region that extends from -5bp to -47bp within the *sodB* promoter (20). At the -5bp, G27 encodes a C while 26695 encodes an A. To determine if this nucleotide difference was important for *sodB* regulation, a "-5bp swap" strain was

Figure 11. Role of the -5bp in sodB *regulation.*

WT G27, WT 26695, and the "-5bp swap" strain were grown as described in the Materials and Methods, and RNA was isolated under iron-replete and iron-depletion shock conditions. RPAs were performed on RNA isolated from 4 biologically independent experiments using *sodB* and *pfr* riboprobes. Data from *sodB* RPAs are presented in Panel A, and data from *pfr* RPAs are presented in Panel B. Each square, diamond, triangle, and circle represent the average fold decrease calculated from three technical repeats with each independent set of RNA for each strain and growth condition combination. Median fold decrease is represented as a bar for each combination, and the dotted-dashed line represents the 2-fold significance cut-off. *p-value of 0.0001. $^{\#}$p-value of 0.006.

Figure 11. Role of the -5bp in sodB *regulation.*



engineered such that the G27 promoter would encode an A at the -5bp position. RPAs were then conducted on RNA isolated from the "-5bp swap" strain along with WT G27 and WT 26695, and results are shown in Fig. 11. While *sodB* expression remained unchanged in G27 under iron depletion shock conditions, a two-fold decrease in *sodB* expression was observed in the "-5bp swap" strain (Fig. 11A). The difference in fold decrease between G27 and the "-5bp swap" was statistically significant with a p-value of 0.006, as was the difference between G27 and 26695 with a p-value of 0.0001. While the fold decrease in *sodB* expression in the "-5bp swap" strain under iron-limited growth conditions did not reach 2-fold, it was consistently higher than its G27 counterpart (data not shown). *apo*-Fur regulation of *pfr* in each of these strains was similar and as expected (17) (Fig. 11B and data not shown). These data suggest that a single nucleotide difference within the *sodB* promoter is at least partially responsible for the difference in regulation of this gene between G27 and 26695.

Comparison of sodB *regulation in various strains of* H. pylori.

Given the differences in *sodB* regulation in G27 and 26695, we wondered if other *H. pylori* strains exhibited *apo*-Fur regulation similar to G27 or 26695. Therefore, we also examined J99 and HPAG1. Analysis of the *sodB* promoter sequences of these two additional strains showed that at the -5bp HPAG1 encodes a C similar to G27, and J99 encodes a G that is different from all other strains (Fig. 9B). Given that the A at the -5bp seems to be crucial for *apo*-Fur regulation of *sodB*, we predicted that these strains would show Fur regulation of *sodB* similar to what was seen with G27. To test this, RPAs were performed on RNA isolated from J99 and HPAG1. As shown in Fig. 10, neither J99 nor

HPAG1 displays the expected decrease in *sodB* expression (20); both behave similarly to G27 (Fig. 10A). However, *pfr* expression (Fig. 10B), basal levels of *fur* expression (Fig. 10C), and *fur* autoregulation (Fig. 10D) are preserved in J99 and HPAG1. Taken together, these data suggest that natural polymorphisms found at the -5bp of the *sodB* promoter in different *H. pylori* strains affect the regulation of *sodB* by *apo*-Fur.

In vitro *binding of Fur to different* sodB *promoters.*

Given that the -5bp in the *sodB* promoter appears to play some role in the *apo*-Fur regulation of *sodB*, we next investigated the direct interaction of *apo*-Fur with the various *sodB* promoters. To assay the binding of *apo*-Fur, we performed Electrophoretic Mobility Shift Assays (EMSAs) and competition studies for each *sodB* promoter (WT G27, "-5bp swap," and WT 26695) using purified Fur under *apo* reaction conditions (20). As shown in Fig. 12, Fur binds to and retards the mobility of each of the three *sodB* promoters, but not the control *rpoB* promoter. Moreover, the addition of homologous unlabeled *sodB* promoter DNA was able to compete for Fur binding with each *sodB* promoter thus confirming specific interaction between Fur and the *sodB* promoters (Fig. 12).

Because *apo*-Fur was able to bind to and shift each of the three *sodB* promoter fragments and because our expression data showed that the -5bp was important for regulation, we reasoned that the various promoter fragments should show differences in their affinity for Fur. To test this, each labeled *sodB* promoter fragment was competed with varying concentrations of its own (homologous) unlabeled promoter fragment as well as with each of the other unlabeled promoter fragments. The success of the

Figure 12. Fur binding to the sodB *promoters.*

EMSAs were performed by incubating various concentrations of purified Fur with radiolabeled fragments of the WT G27, "-5bp swap," and WT 26695 *sodB* promoters as well as the negative control promoter, *rpoB*, as detailed in the Materials and Methods. In the first four lanes, the Fur concentrations are indicated by the triangle from highest to lowest and range from 1.07μg/mL to 0.026μg/mL. A no protein control for each promoter is found in the fifth lanes. The last lane shows the 100x cold (unlabeled) competition control for each promoter fragment, which were each performed with the highest concentration of Fur (1.07μg/mL). Fur exhibits specific interaction with each of the *sodB* promoters, and no interaction with the *rpoB* promoter except for very little non-specific binding at the highest Fur concentration. These data are representative of multiple independent EMSA experiments.

Figure 12. Fur binding to the sodB *promoters.*

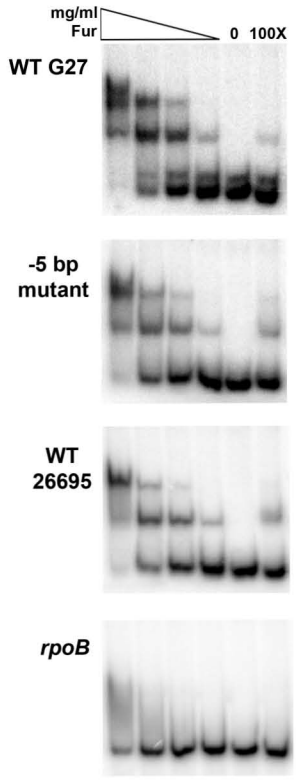
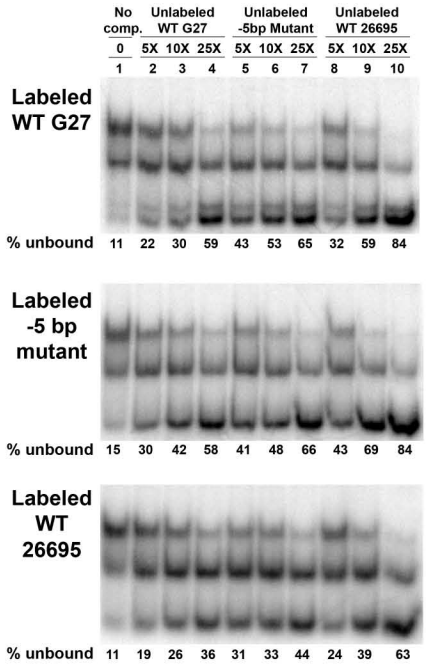


Figure 13. Competitive Binding Studies.

To assess the relative affinity of Fur for each of the sodB promoter fragments (WT G27, "-5bp swap," and WT 26695), Fur was incubated with each radiolabeled promoter and 5x, 10x, or 25x the amount of homologous or heterologous unlabeled sodB promoter fragments as described in the Materials and Methods. For each labeled promoter, lane one contains a no competition control. Lanes two to four, five to seven, and eight to ten contain the competition EMSAs with unlabeled WT G27, "-5bp swap," and WT 26695 sodB fragments, respectively. The percent of labeled promoter that is outcompeted and remains unbound in each lane is given below each image. These data are representative of multiple independent experiments.

Figure 13. Competitive Binding Studies.



competition was then measured by quantitating the percent of unbound probe resulting from each competition reaction such that $PD_{P32} + D \longleftrightarrow PD + D_{P32}$, where P = Fur, D_{P32} = labeled DNA, and D = cold competitor. As shown in Fig. 13, the various promoter fragments showed differences in affinity such that 26695 ≥ -5bp > G27. In all cases, the 26695 and -5bp promoter were better able to compete for Fur binding as the largest percentages of unbound labeled promoter fragment are observed with these two promoters in comparison to the WT G27 *sodB* promoter. Taken together with the expression data, these data indicate that the -5bp is important for Fur interaction at the *sodB* promoter.

Discussion

Given how panmictic *H. pylori* is, it is not surprising that genes may be regulated differently in different strains. Indeed, there have been several instances of this reported in the literature in recent years involving acid-response and CrdRS (29), *vacA* regulation (2), virulence gene regulation *in vivo* (25), and *cagA* and *vacA* expression in response to salt (24). In addition, a single nucleotide polymorphism upstream of the Fur-box was found to alter Fur regulation of *IrgA* in two different strains of *E. coli* (30) indicating that there may be more to Fur regulation in other organisms than just binding at the recognition sequence. This study adds to that body of knowledge and is the first to explore the differences in Fur regulation among different strains of *H. pylori*.

apo-Fur regulation remains a unique form of Fur regulation found only in *H. pylori*. Additionally, our understanding of this type of regulation is currently limited as only two *apo*-Fur repressed genes, *sodB* (20) and *pfr* (17), have been characterized. Here

we present evidence that *H. pylori* shows strain specific differences in *sodB apo*-regulation that are partially controlled by a natural polymorphism found at the -5bp of the *sodB* promoter. Alteration of this single nucleotide in the G27 promoter to resemble the residue found in 26695 resulted in alteration of G27 *sodB* regulation that mimicked regulation seen in 26695. Based on this observation, we accurately predicted that two other commonly used strains of *H. pylori*, J99 and HPAG1, would show altered *sodB* regulation since they each encode a different nucleotide at the -5 position within the *sodB* promoter.

The importance of the -5bp within the *sodB* promoter is further supported by our EMSA competition data. At low concentrations of competitor DNA, the "-5bp swap" promoter is able to bind to *apo*-Fur with an affinity similar to WT 26695 while WT G27 exhibits weaker binding. At higher concentrations of competitor, the affinity of the "-5bp swap" promoter for *apo*-Fur is still greater than WT G27 but slightly less than WT 26695. Thus, it appears that strain specific regulation of *sodB* is due to differences in the affinity of Fur for the various promoters and that natural polymorphisms at the -5bp are largely responsible for this differential regulation.

The significance of the *sodB* polymorphism in *H. pylori* fitness, especially *in vivo,* is currently unclear. However, the affinity of *apo*-Fur for the *sodB* promoter in 26695 was reported to be relatively weak (K_d = 260nM) (20), and based upon our competition data it is likely even weaker in G27. As Ernst, et al. suggested, a weak affinity between *apo*-Fur and the *sodB* promoter makes physiological sense, as SodB is the only defense *H. pylori* has against superoxide radical damage (20, 31). Therefore, it would be ill-advised to repress *sodB* under conditions where any iron is still available, since iron-

catalyzed oxidative damage could still be possible (20). In keeping with this notion, some strains of *H. pylori* may have evolved to either inactivate *apo*-Fur regulation of *sodB,* or to weaken repression by decreasing the Fur/*sodB* binding affinity. Also of note, as shown in Fig. 8, in the absence of Fur, iron chelation results in slight increases in *sodB* (and *pfr*) perhaps suggesting the presence of additional regulatory proteins that ensure proper expression of this critical factor.

Furthermore, it is interesting to speculate that strains, which possess sequences similar to 26695, might actually show decreased *in vivo* fitness due to decreased expression of *sodB* in the iron limited environment of the stomach. Analysis of the *sodB* promoter sequence in the efficient gerbil colonizing strain B128 (isolate 7.13) (22) revealed that B128, similar to G27, encodes a C at the -5bp (data not shown). Therefore, studies could potentially be designed with this strain that would allow for the determination of whether direct *apo*-Fur regulation of *sodB* provides a competitive advantage to *H. pylori in vivo*.

Currently, little is understood about the sequences recognized by *H. pylori* Fur that dictate binding of the protein at target promoters. This is true of both iron-bound and *apo* forms of Fur. In *E. coli*, Fur binding has been shown to involve recognition of a well-conserved consensus sequence called a Fur Box. This Fur Box consists of two 9bp inverted repeat sequences separated by a single A nucleotide to create a 19bp palindromic sequence as follows: GATAATGATAATCATTATC (12). This sequence can also be interpreted as a series of three hexameric repeats of NATA/TAT (21). However, in *H. pylori* this *E. coli* Fur Box is not conserved, and consensus is currently ill-defined. For iron-bound Fur regulation, the binding sequence occurs in A/T-rich regions in the target

promoter oftentimes with repeats of AAT (13-15, 17, 36). There is no defined consensus sequence for *apo*-Fur binding given that the two promoters of the known *apo*-Fur regulated genes, *pfr* and *sodB*, share only minimal homology (17, 20). In an organism that has about 60% A/T residues in its genome, a Fur Box consensus sequence that is comprised of mainly these two nucleotides does not seem to be an ideal approach for Fur regulation. Rather, in *H. pylori* it is perhaps more plausible that both iron-bound and *apo*-Fur recognize unique DNA structures that are required for proper regulation of their target genes. The work presented here is the first to define a residue that is important for *apo*-Fur binding to the *sodB* target promoter. Future work from our group will focus on elucidating binding residues important for both iron-bound and *apo*-Fur regulation with the hope that continued exploration of Fur regulation will provide greater understanding into the complexity of gene regulation in this important human pathogen.

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References

1. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.

2. **Ayala, G., L. Chihu, G. Perales, G. Fierros-Zarate, L. M. Hansen, J. V. Solnick, and J. Sanchez.** 2004. Quantitation of *H. pylori* cytotoxin mRNA by real-time RT-PCR shows a wide expression range that does not correlate with promoter sequences. Microb Pathog **37:**163-7.

3. **Bereswill, S., F. Lichte, T. Vey, F. Fassbinder, and M. Kist.** 1998. Cloning and characterization of the *fur* gene from *Helicobacter pylori*. FEMS Microbiol Lett **159:**193-200.

4. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis **161:**626-33.

5. **Bury-Mone, S., J. M. Thiberge, M. Contreras, A. Maitournam, A. Labigne, and H. De Reuse.** 2004. Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. Mol Microbiol

53:623-38.

6. **Carpenter, B. M., T. K. McDaniel, J. M. Whitmire, H. Gancz, S. Guidotti, S. Censini, and D. S. Merrell.** 2007. Expanding the *Helicobacter pylori* genetic toolbox: modification of an endogenous plasmid for use as a transcriptional reporter and complementation vector. Appl Environ Microbiol **73:**7506-14.

7. **Copass, M., G. Grandi, and R. Rappuoli.** 1997. Introduction of unmarked mutations in the *Helicobacter pylori vacA* gene with a sucrose sensitivity marker. Infect Immun **65:**1949-52.

8. **Cormack, B. P., R. H. Valdivia, and S. Falkow.** 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173:**33-8.

9. **Corpet, F.** 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res **16:**10881-90.

10. **Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, and R. Rappuoli.** 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A **90:**5791-5.

11. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-5.

12. **de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J Bacteriol **169:**2624-30.

13. **Delany, I., R. Ieva, A. Soragni, M. Hilleringmann, R. Rappuoli, and V. Scarlato.** 2005. In vitro analysis of protein-operator interactions of the NikR and fur metal-responsive regulators of coregulated genes in *Helicobacter pylori*. J Bacteriol **187:**7703-15.

14. **Delany, I., A. B. Pacheco, G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. Iron-dependent transcription of the *frpB* gene of *Helicobacter pylori* is controlled by the Fur repressor protein. J Bacteriol **183:**4932-7.

15. **Delany, I., G. Spohn, A. B. Pacheco, R. Ieva, C. Alaimo, R. Rappuoli, and V. Scarlato.** 2002. Autoregulation of *Helicobacter pylori* Fur revealed by functional analysis of the iron-binding site. Mol Microbiol **46:**1107-22.

16. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2003. An anti-repression Fur operator upstream of the promoter is required for iron-mediated

transcriptional autoregulation in *Helicobacter pylori*. Mol Microbiol **50:**1329-38.

17. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. Mol Microbiol **42:**1297-309.

18. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin Microbiol Rev **10:**720-41.

19. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1989. *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect Immun **57:**1119-25.

20. **Ernst, F. D., G. Homuth, J. Stoof, U. Mader, B. Waidner, E. J. Kuipers, M. Kist, J. G. Kusters, S. Bereswill, and A. H. van Vliet.** 2005. Iron-responsive regulation of the *Helicobacter pylori* iron-cofactored superoxide dismutase SodB is mediated by Fur. J Bacteriol **187:**3687-92.

21. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 1998. Binding of the fur (ferric uptake regulator) repressor of Escherichia coli to arrays of the GATAAT sequence. J Mol Biol **283:**537-47.

22. **Franco, A. T., D. A. Israel, M. K. Washington, U. Krishna, J. G. Fox, A. B. Rogers, A. S. Neish, L. Collier-Hyams, G. I. Perez-Perez, M. Hatakeyama, R.**

Whitehead, K. Gaus, D. P. O'Brien, J. Romero-Gallo, and R. M. Peek, Jr. 2005. Activation of beta-catenin by carcinogenic *Helicobacter pylori*. Proc Natl Acad Sci U S A **102:**10646-51.

23. **Gancz, H., S. Censini, and D. S. Merrell.** 2006. Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen *Helicobacter pylori*. Infect Immun **74:**602-14.

24. **Gancz, H., K. R. Jones, and D. S. Merrell.** 2008. Sodium chloride affects *Helicobacter pylori* growth and gene expression. J Bacteriol **190:**4100-5.

25. **Gieseler, S., B. Konig, W. Konig, and S. Backert.** 2005. Strain-specific expression profiles of virulence genes in *Helicobacter pylori* during infection of gastric epithelial cells and granulocytes. Microbes Infect **7:**437-47.

26. **Gutteridge, J. M., L. Maidt, and L. Poyer.** 1990. Superoxide dismutase and Fenton chemistry. Reaction of ferric-EDTA complex and ferric-bipyridyl complex with hydrogen peroxide without the apparent formation of iron(II). Biochem J **269:**169-74.

27. **Mehta, N., J. W. Olson, and R. J. Maier.** 2003. Characterization of *Helicobacter pylori* nickel metabolism accessory proteins needed for maturation of both urease and hydrogenase. J Bacteriol **185:**726-34.

28. **Oh, J. D., H. Kling-Backhed, M. Giannakis, J. Xu, R. S. Fulton, L. A. Fulton, H. S. Cordum, C. Wang, G. Elliott, J. Edwards, E. R. Mardis, L. G. Engstrand, and J. I. Gordon.** 2006. The complete genome sequence of a chronic atrophic gastritis *Helicobacter pylori* strain: evolution during disease progression. Proc Natl Acad Sci U S A **103:**9999-10004.

29. **Pflock, M., S. Muller, and D. Beier.** 2007. The CrdRS (HP1365-HP1364) two-component system is not involved in ph-responsive gene regulation in the *Helicobacter pylori* Strains 26695 and G27. Curr Microbiol **54:**320-4.

30. **Rashid, R. A., P. I. Tarr, and S. L. Moseley.** 2006. Expression of the Escherichia coli IrgA homolog adhesin is regulated by the ferric uptake regulation protein. Microb Pathog **41:**207-17.

31. **Seyler, R. W., Jr., J. W. Olson, and R. J. Maier.** 2001. *Superoxide dismutase*-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. Infect Immun **69:**4034-40.

32. **Spiegelhalder, C., B. Gerstenecker, A. Kersten, E. Schiltz, and M. Kist.** 1993. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. Infect Immun **61:**5315-25.

33. **Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee, and S. Falkow.** 2003. Gene expression profiling of Helicobacter pylori reveals a growth-phase-dependent switch in virulence gene expression. Infect Immun **71:**2643-55.

34. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

35. **van Amsterdam, K., A. H. van Vliet, J. G. Kusters, and A. van der Ende.** 2006. Of microbe and man: determinants of *Helicobacter pylori*-related diseases. FEMS Microbiol Rev **30:**131-56.

36. **van Vliet, A. H., J. Stoof, S. W. Poppelaars, S. Bereswill, G. Homuth, M. Kist, E. J. Kuipers, and J. G. Kusters.** 2003. Differential regulation of amidase- and formamidase-mediated ammonia production by the *Helicobacter pylori* fur repressor. J Biol Chem **278:**9052-7.

37. **Wang, G., R. C. Conover, A. A. Olczak, P. Alamuri, M. K. Johnson, and R. J. Maier.** 2005. Oxidative stress defense mechanisms to counter iron-promoted DNA damage in *Helicobacter pylori*. Free Radic Res **39:**1183-91.

Chapter Four

Mutagenesis of Conserved Amino Acids of Helicobacter pylori *Fur Reveals Residues Important for Function*

Submitted as: **Carpenter, B.M., Gancz, H., Benoit, S.L., Evans, S., Michel, S.L., Maier, R.J., and Merrel, D.S.** Mutagenesis of Conserved Amino Acids of *Helicobacter pylori* Fur Reveals Residues Important for Function. J. Bacteriology *submitted*.

The work presented in this chapter is the sole work of B.M. Carpenter with the following exceptions: H. Gancz assisted in protein purification and figure generation, S.L. Benoit and R.J. Maier performed the iron binding studies, S. Evans and S.L. Michel performed the circular dichroism and thermal denaturation analyses.

Abstract

The Ferric Uptake Regulator (Fur) of the medically important pathogen, *Helicobacter pylori*, is unique in that it has been shown to function as a repressor both in the presence of an Fe^{2+} cofactor as well as in its *apo* (non-Fe^{2+}-bound) form. However, virtually nothing is known concerning the amino acid residues that are important for Fur functioning. Therefore, mutations in six conserved amino acid residues of *H. pylori* Fur were constructed and analyzed for their impact on both iron-bound and *apo* repression as well as on autoregulation. In addition, accumulation of the mutant proteins, protein secondary structure, DNA binding ability, iron binding capacity, and the ability to form

higher order structures were also examined for each mutant protein. While none of the mutated residues completely abrogated the function of Fur, we were able to identify residues that were critical for both iron-bound and *apo*-Fur repression. One mutation, V64A, did not alter regulation of any target genes. However, each of the five remaining mutations showed an affect on either iron bound or *apo* regulation. Of these, H96A, E110A, and E117A altered iron-bound Fur regulation and were all shown to influence iron binding though to different extents. Additionally, H96A was shown to alter Fur oligomerization and E110A was shown to impact oligomerization and DNA binding. Conversely, H134A exhibited changes in *apo*-Fur regulation that were the result of alterations in DNA binding. Although E90A exhibited alterations in *apo*-Fur regulation, this mutation did not affect any of the assessed protein functions. This study is the first in *H. pylori* to analyze the role of specific amino acid residues of Fur for function and continues to highlight the complexity of Fur regulation in this organism.

Introduction

Infecting over half of the world's population, the Gram negative, microaerophilic bacterium, *Helicobacter pylori*, is a successful pathogen that is well adapted to its chosen niche within the human gastric mucosa (27). Infection with *H. pylori* usually occurs in early childhood and can last throughout a lifetime unless treated with a specific antibiotic regimen (12). Despite the chronic nature of infection, *H. pylori* infections are largely asymptomatic and cause more serious disease in a small percentage of infected individuals; disease states range from gastritis and peptic ulcer disease to two forms of gastric cancer, gastric adenocarcinoma and mucosa-associated lymphoid tissue

lymphoma (12). Given the sheer number of infected individuals, the chronic nature of infection and the potential for severe disease outcome, *H. pylori* potentiates a large medical burden worldwide.

The success of this organism as a pathogen can be attributed to a myriad of factors that help *H. pylori* respond and adapt to the changing environment within the stomach. One such factor that plays a critical role in helping *H. pylori* maintain iron homeostasis is the **F**erric **U**ptake **R**egulator (Fur) (50). Iron is a critical nutrient for virtually all forms of life due to its utilization as an enzymatic co-factor and in the electron transport chain. However, a delicate balance must be maintained between having enough iron to support life and having too much iron; excess iron can result in DNA damage and cellular death through Fenton chemistry and the formation of hydroxyl radicals. Thus, it is no surprise that iron homeostasis is crucial for the survival of all organisms that utilize this nutrient.

Fur is a small protein of approximately 150 amino acids with a size ranging from 15 to17 kDa (16). Fur functions as a transcriptional regulator for genes involved in iron uptake and storage in numerous bacterial species (16). Classically, this regulation occurs under conditions of high iron availability when Fur is bound by its ferrous (Fe^{2+}) iron co-factor and is subsequently able to dimerize (5). Iron-bound Fur dimers then bind to specific regions of DNA, called "Fur-boxes," in the promoters of target genes. This in turn occludes the binding site for RNA Polymerase (RNAP) thus, repressing transcription (5). Iron-bound Fur regulation occurs in this manner in *H. pylori*. In addition, in *H. pylori*, Fur has been found to repress an additional set of genes in the absence of its iron co-factor in what is termed *apo*-Fur regulation (9, 26). Binding at *apo*-Fur boxes occurs

under iron limited conditions, and again Fur binding prevents the binding of RNAP. *apo*-Fur regulation has only been definitively shown to occur in *H. pylori*, although microarray studies suggest it may also occur in *Campylobacter jejuni* (33) and *Desulfovibrio vulgaris* Hildenborough (8). Compared to what is known about iron-bound Fur regulation, little is currently understood concerning *apo*-Fur regulation.

In the vast majority of bacteria that utilize Fur, *fur* expression is autoregulatory; under conditions of abundant iron, iron-bound Fur binds to Fur-boxes within the *fur* promoter and represses its own transcription. In this manner, Fur can be thought of as a rheostat that responds to changes in iron availability and adjusts *fur* expression accordingly (25). In *H. pylori*, Fur is autoregulatory, but autoregulation is more complex than the basic scheme presented above. Not only does iron-bound Fur bind to and repress *fur* transcription, but *apo*-Fur has also been shown to bind to the *fur* promoter and activate *fur* transcription under low iron conditions (24, 25). Thus, there is an intimate interplay between iron-bound and *apo*-Fur, and both forms of the protein have important roles in properly maintaining expression of this crucial regulatory protein.

While not an essential gene (10, 17), Fur is important for colonization in both gerbil (31) and murine (13) models of *H. pylori* infection. The iron-bound form of Fur represses a large group of genes (21, 41) including several involved in iron uptake in this organism [FrpB (23, 26, 50) and FeoB (50)]. In addition, genes that are not directly involved in iron homeostasis, like the aliphatic amidase, *amiE*, are also regulated by iron-bound Fur (15, 49). AmiE functions in nitrogen metabolism and helps to combat pH mediated stress in the cell (45). Currently, there are approximately 16 genes believed to comprise the *apo*-Fur repression regulon (28). Two of these have been shown to be

directly repressed by *apo*-Fur through DNase Footprinting and/or DNA binding analysis; *sodB*, *H. pylori*'s only superoxide dismutase, and *pfr*, a non-heme iron containing ferritin, are both repressed by Fur in the absence of iron (9, 14, 15, 26, 29).

Fur is a well studied protein, and several mutational analyses performed on Fur from different model organisms have provided functional insight. For instance, mutational analysis of 12 His and 4 Cys residues in *Escherichia coli* Fur identified four critical residues: H32, H117, C92, and C95 and identified C92 and C95 as metal binding ligands (19). Nuclear magnetic resonance (NMR) studies identified, His33 and His132 as metal binding ligands (43). Mutagenesis of *Vibrio cholerae* Fur showed that residues H90 and D113, were important for Fur function (35). H90 lies in a highly conserved motif (HHDH) that is predicted to be involved in iron binding in *E. coli* (4, 35, 43). In *Pseudomonas aeruginosa* Fur, A10 and H86 are both critical for Fur regulation (7, 32). *P. aeruginosa* Fur, like that of *E. coli* and *V. cholerae*, also contains the highly conserved HHDH region (*P. aeruginosa* residues 86-89). Mutation of these residues showed that while H86 and D88 were partially dispensable, H87 and H89 were essential for Fur function (40). However, unlike *E. coli*, which contains Cys residues that are required for metal binding and function, *P. aeruginosa* Fur lacks these essential Cys residues (40). This fact was affirmed when the crystal structure of *P. aeruginosa* Fur was resolved (42). The importance of the HHDH region was also confirmed by the crystal structure as H86 and D88 were shown to be involved in coordinating one of the two metal ions in Fur, and H89 was involved in coordinating the other (42). The full complement of amino acids that serve as metal binding ligands for *H. pylori* Fur have not been resolved; however, D98 and H99 within the conserved HHDH region of *H. pylori* Fur have been identified as

important for Fur autoregulation and DNA binding suggesting that they may serve as metal binding ligands (24).

Given how well characterized Fur structure function relationships are in other organisms, it is surprising that virtually no such analysis has been applied to *H. pylori* Fur to help identify amino acid residues critical for Fur functioning. This is particularly surprising given the fact that in this organism, Fur functions as a classical iron-bound repressor and as a unique *apo* repressor. Herein, we present the first structure function analysis of *H. pylori* Fur. The effects of six site specific amino acid mutations in conserved residues of Fur were analyzed for their affects on iron-bound and *apo* regulation. Moreover, we analyzed the role of these residues on Fur autoregulation, iron binding, protein stability, and the ability to form higher order structures.

Materials and Methods

Bacterial strains and growth

All bacterial strains and plasmids utilized in this study are listed in Table 7, and all primers are shown in Table 8. All *H. pylori* strains were maintained as frozen stocks in brain heart infusion broth (BD) supplemented with 20% glycerol (EMD Chemicals, Inc.) and 10% fetal bovine serum (Gibco) at -80ºC, and all *E. coli* strains were maintained as frozen stocks (-80ºC) in LB broth (MO Bio) supplemented with 40% glycerol. *H. pylori* strains were grown on horse blood agar (HBA) plates consisting of 4% Columbia agar base (Neogen Corporation), 5% defibrinated horse blood (HemoStat Laboratories, Dixon, CA), 0.2% β-cyclodextrin (Sigma), 8μg/mL amphotericin B (Amresco), 2.5U/mL polymyxin B (Sigma), 5μg/mL cefsulodin (Sigma), 5μg/mL

Table 7. Plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pDSM327	pET21A::26695 *fur*	(2)
pDSM361	pGemT-Easy::Fur V64A	This study
pDSM378	pGemT-easy::Fur H134A	This study
pDSM379	pGemT-easy::Fur E90A	This study
pDSM380	pGemT-easy::Fur E110A	This study
pDSM383	pGemT-easy::Fur H96A	This study
pDSM385	pGemT-easy::Fur E117A	This study
pDSM430	pET21A:: 26695 Fur	(14)
pDSM655	pGemT-easy::promoterless Fur H134A	This study
pDSM656	pGemT-easy::promoterless Fur E90A	This study
pDSM657	pGemT-easy::promoterless Fur E110A	This study
pDSM658	pGemT-easy::promoterless Fur H96A	This study
pDSM659	pGemT-easy::promoterless Fur E117A	This study
pDSM660	pGemT-easy::promoterless Fur V64A	This study
pDSM678	pET21A::promoterless Fur V64A	This study
pDSM679	pET21A::promoterless Fur E90A	This study
pDSM680	pET21A::promoterless Fur H96A	This study
pDSM681	pET21A::promoterless Fur E110A	This study
pDSM682	pET21A::promoterless Fur E117A	This study
pDSM683	pET21A::promoterless Fur H134A	This study
H. pylori strains		
G27	WT *H. pylori*	(18)
DSM300	G27 Δ*fur*::*cat,* Cm^r	(15)
DSM391	G27 Δ*fur*::*kan-sacB*, Kan^r Suc^s	(14)
DSM395	G27 Fur H134A, Suc^r, Kan^s	This study
DSM396	G27 Fur E90A, Suc^r, Kan^s	This study
DSM397	G27 Fur E110A, Suc^r, Kan^s	This study
DSM399	G27 Fur H96A, Suc^r, Kan^s	This study
DSM400	G27 Fur E117A, Suc^r, Kan^s	This study
DSM402	G27 Fur V64A, Suc^r, Kan^s	This study
E. coli strains		
DSM365	BL21 DE3 Rosetta/pLysS Δ*fur*, Kan^r, Cm^r	(14)
DSM431	BL21Δ*fur* (pDSM430) Amp^r, Cm^r, Kan^r	(14)
DSM686	BL21 DE3 Rosetta/pLysS Δ*fur*, (pDSM678), Amp^r, Kan^r, Cm^r	This study

DSM687	BL21 DE3 Rosetta/pLysS Δ*fur*, (pDSM679), Amp^r, Kan^r, Cm^r	This study
DSM688	BL21 DE3 Rosetta/pLysS Δ*fur*, (pDSM680), Amp^r, Kan^r, Cm^r	This study
DSM689	BL21 DE3 Rosetta/pLysS Δ*fur*, (pDSM681), Amp^r, Kan^r, Cm^r	This study
DSM690	BL21 DE3 Rosetta/pLysS Δ*fur*, (pDSM682), Amp^r, Kan^r, Cm^r	This study
DSM691	BL21 DE3 Rosetta/pLysS Δ*fur*, (pDSM683), Amp^r, Kan^r, Cm^r	This study

Table 8. Primers used in this study

Primer[a]	Sequence (5'-3')[b]	Reference
SOE primers		
FurCF1 (XbaI)	TCTAGAAAGGCTCACTCTACCCTATT	(15)
FurCR (SalI)	GTCGACAAGACTTTCACCTGGAAACGC	(15)
V64A SOE R2	CAAAATGCGATA**GGC**TGAAGAAATG	This study
V64A SOE F3	CATTTCTTCA**GCC**TATCGCATTTTG	This study
E90A SOE R2	CTTTAGCCGCAAT**GGC**ATAGCGCCGAC	This study
E90A SOE F3	GTCGGCGCTAT**GCC**ATTGCGGCTAAAG	This study
H96A SOE R2	GATGTGATCATG**GGC**TTCTTTAGCC	This study
H96A SOE F3	GGCTAAAGAA**GCC**CATGATCACATC	This study
E110A SOE R2	GGTCTGCAAA**GGC**AATGATCTTACC	This study
E110A SOE F3	GGTAAGATCATT**GCC**TTTGCAGACC	This study
E117A SOE R2	CATTCTGGCGGTT**GGC**AATTTCAGG	This study
E117A SOE F3	CCTGAAATT**GCC**AACCGCCAGAATG	This study
H134A SOE R2	CATTTTCATGTC**AGC**GCTAATCAGC	This study
H134A SOE F3	GCTGATTAGC**GCT**GACATGAAAATG	This study
Promoterless Expression primers		
HPFurMt_expression_F (NdeI)	CAT*ATG*AAAAGATTAGAAACTTTGGAATCTATTTT	This study
HPFurMt_expression_R (XhoI)	CTCGAG*TTA*TTAATATTCACTCTCTTGG	This study
RPA primers		
amiE-RPA-F	GGTTTGCCTGGGTTGGAT	(31)
amiE-RPA-R	GATTTTGCGGTATTTTTG	(31)

pfr-RPA-F	GCGGCTGAAGAATACGAG	(15)
pfr-RPA-R	CTGATCAGCCAAATACAA	(15)
fur RPA F	GAGCGCTTGAGGATGTCTATC	(15)
fur RPA R	GTGATCATGGTGTTCTTTAGC	(15)
EMSA primers		
amiE EMSA-F	CCTCATTTTCAGAAATAGG	This study
amiE EMSA-R	GTGTAAATGGATCTATAATG	This study
pfr EMSA-F	CCCCTTTTTACCTAATTCTC	This study
pfr EMSA-R	CCTTATTCACTTGTTCGTTTAG	This study
rpoB EMSA-F	CCAAAGAGGGTAAAGAGAGCG	(14)
rpoB EMSA-R	CCTCTCCATCGCTTCTCTAAC	(14)

[b]Important restriction sites are included in parentheses

[a]Restriction endonuclease sites are underlined, Ala mutation mucleotides are in bold type, and ATG start codons or additional TAA (TTA in the reverse primer) stop codons are italicized.

trimethoprim (Sigma), and 10μg/mL vancomycin (Amresco). Liquid cultures of *H. pylori* were grown at 37ºC shaking at 100 rpm in brucella broth (BB) (Neogen Corporation) that was supplemented with 10% fetal bovine serum (FBS) and 10μg/mL vancomycin. Liquid and plate cultures of *H. pylori* were grown under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) in gas evacuation jars generated using an Anoxomat gas evacuation and replacement system (Spiral Biotech). *E. coli* strains were grown on either LB Agar plates (MO Bio) or in LB broth (MO Bio) liquid cultures. Bacterial cultures were supplemented with the following antibiotics as noted in Table 7: ampicillin (Amp) (USB Corporation) 100μg/mL, kanamycin (Kan) (Gibco) 25μg/mL, and/or chloramphenicol (Cm) (EMD Chemicals, Inc.) at 8μg/mL for *H. pylori* and 25μg/mL for *E. coli*. In addition, where needed 5% sucrose (Sigma) was added to HBA plates as described elsewhere in the Materials and Methods and as noted in Table 7. Exponential phase cultures were grown for 20 hours. All *H. pylori* strains used in this study are derivatives of the wild-type (WT) strain, G27 (18).

Creation of Site Specific Fur Mutations

Residues, V64, E90, H96, E110, E117, and H134, were changed to an alanine using Splicing by Overlap Extension (SOE) PCR using the primer pairs listed in Table 8. Briefly, the FurCF1 (XbaI) and individual mutation specific SOE R2 primers were used to PCR amplify the region upstream of the WT G27 *fur* promoter through to and including the site specific mutation. Additionally, the mutation specific SOE F3 and FurCR (SalI) primers were used to PCR amplify from the mutation site to beyond the end of the *fur* coding sequence. These fragments were gel purified using the QIAquick Gel

Extraction Kit (Qiagen), and 150ng of each purified product was combined in the SOE reaction and amplified using the FurCF1 (XbaI) and FurCR (SalI) primers. The resultant PCR products were subsequently cloned into pGEM T-Easy (Promega) and were sequenced with the FurCR (SalI) primer to ensure that the desired mutations were present. These plasmids were named pDSM361, pDSM378, pDSM379, pDSM380, pDSM383, and pDSM385 and carry the V64A, H134A, E90A, E110A, H96A, and E117A *fur* mutations, respectively.

Each of these plasmids was then naturally transformed into *H. pylori* strain DSM391 in which the entire *fur* coding sequence is replaced with the counter selectable *kan-sacB* cassette (14). Briefly, double crossover homologous recombination of the site specific *fur* mutations results in replacement of the *kan-sacB* cassette with the mutant construct; therefore, transformants were selected on HBA plates containing 5% sucrose. Kan sensitivity for each was confirmed by streaking on HBA plates containing 25μg/mL Kan. Proper integration was confirmed by PCR amplification of the constructs with the FurCF1 (XbaI) and FurCR (SalI) primer pair (923bp fragment) followed by sequencing of this product with the FurCR (SalI) primer. The following strains carry the H134A, E90A, E110A, H96A, E117A, and V64A *fur* mutations, respectively: DSM395, DSM396, DSM397, DSM399, DSM400, and DSM402.

RNase Protection Assays (RPAs)

RPAs were performed as previously described (14, 15). Briefly, liquid cultures were inoculated for each strain and grown in BB supplemented with 10% FBS (iron abundant) media into exponential phase. One half of the each culture was then removed

for RNA isolation, and 200μM of the iron chelator, 2,2'-dipyridyl (dpp), was added to the remaining half of each culture to create an iron depletion shock environment (41). The cultures were maintained for an additional hour prior to RNA isolation as previously described (47). Integrity of RNA was confirmed by visualization on agarose gels, and 1.5μg of RNA was used in each RPA with riboprobes for *amiE*, *pfr*, and *fur*. Riboprobes were generated using the primer pairs listed in Table 8, 50μCi [^{32}P]UTP (Perkin-Elmer), and the Maxiscript kit (Applied Biosystems). RPA reactions were generated with the RPA III kit (Applied Biosystems), and the reactions were resolved on 5% acrylamide-1X Tris-borate-EDTA-8M urea denaturing gels. Afterwards, gels were exposed to phosphor screens, which were scanned using a FLA-5100 multifunctional scanner (FujiFilm). Data were analyzed/quantitated using the Multi-Gauge software (version 3.0, FujiFilm). Four independent biological repeats of each experiment were conducted.

Western Blot Analysis

Western blot analysis was performed on bacterial cell lysates made from the same liquid cultures (pre and post iron chelation shock) used for RNA isolation in the RPA experiments. 1.0mL of each Fur mutant culture was pelleted and washed twice with 1x phosphate buffered saline (PBS). Cells were lysed with 200μL of lysis buffer (150mM NaCl, 50mM Tris-HCl, pH 8.0, 5mM EDTA, 1% sodium dodecyl sulfate (SDS), 10% glycerol containing one Complete Mini Protease Inhibitor Cocktail Tablet (Roche) per 50mL lysis buffer). Lysates were centrifuged to remove debris, and the amount of protein in each lysate was quantitated using the BCA Protein Assay Kit (Pierce). 6μg of total protein was combined with 5μL of 5x Laemmli sample buffer. Proteins were

separated on a 20% SDS-PAGE gel, and a semi-dry transfer apparatus (OWL, ThermoScientific) was used to transfer the protein to nitrocellulose membranes. Membranes were probed with a 1:200 dilution of anti-*H. pylori* Fur polyclonal rabbit sera followed by a 1:20,000 dilution of HRP conjugated bovine anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). The Super Signal West Pico Chemiluminescent Substrate kit (ThermoScientific/Pierce) and a LAS-3000 Intelligent Dark Box with LAS-3000 Lite capture software (FujiFilm) were used to detect the proteins. Quantification and analysis of the Fur bands were performed using Multi-Gauge software (version 3.0, FujiFilm), and three independent biological repeats of the Western blot analysis were performed.

The anti-*H. pylori* Fur polyclonal rabbit sera was generated at the Pocono Rabbit Farm and Laboratory, Inc. (PRF&L) using the "Rabbit Quick Draw Protocol" with a one month protocol extension. The rabbits were immunized with purified *H. pylori* Fur and PRF&L's immune stimulator on days 0, 7, 14, and 35. Terminal bleeds were performed on day 56, and this sera was utilized in these studies.

Creation of Mutant Fur Expression Strains and Protein Purification

Each of the mutant *fur* coding sequences was amplified from its respective pGEM T-Easy clone using the FurMt_expression_F (NdeI) and FurMt_expression_R (XhoI) primer pair (Table 8). The FurMt_expression_R (XhoI) primer contains an additional stop codon to ensure that translation is terminated appropriately in the expression system (see Table 8). The promoterless *fur* coding sequence PCR products were subsequently cloned into the pGEM T-Easy vector generating plasmids, pDSM655 - pDSM660 for the

H134A, E90A, E110A, H96A, E117A, and V64A mutations, respectively. Constructs were confirmed by EcoRI (Invitrogen) restriction digestion and by sequencing. Confirmed constructs were double digested with NdeI and XhoI (New England Biolabs), and the purified 453bp mutant *fur* fragment was ligated into the appropriately digested and purified pET21A (2) expression vector (pDSM327) such that isopropyl-beta-D-thiogalactopyranoside (IPTG) could be used to induce expression of each Fur mutant protein. These plasmids were named pDSM678 - pDSM683 and contain the V64A, E90A, H96A, E110A, E117A, and H134A Fur mutations, respectively. Each Fur mutant expression plasmid was next transformed into DSM365 (14), which is a Δ*fur E. coli* BL21 DE3 Rosetta/pLysS expression strain, and transformants were selected on plates containing Amp, Kan, and Cm. These transformations resulted in the creation of strains DSM686 to DSM691 containing the pDSM678 to pDSM683 plasmids, respectively. The expression plasmids were again confirmed in this strain background by sequencing with both the HPFurMt_expression_F (NdeI) and HPFurMt_expression_R (XhoI) primers.

Purification of the Fur mutants along with WT Fur from the previously characterized expression strain, DSM431 (14), was performed exactly as described previously (14). The peak fractions for each protein were combined and stored with an equal volume of protein storage buffer (buffer C plus 50% glycerol) at -20ºC. Protein concentrations were determined using the Pierce BCA Protein Assay kit. In addition, for two portions of each protein, Amicon Ultra Centrifugal Filter Devices (Millipore) were used to remove the protein storage buffer and replace it with either *apo*-Binding Buffer with 50% glycerol or $MnCl_2$-Binding Buffer with 50% glycerol for later use in the electrophoretic mobility shift assay analysis.

Circular Dichroism (CD) Studies

CD spectra of WT and mutant Fur proteins were collected on a Jasco-810 spectropolarimeter. Spectra were acquired in a 5mm path length cell at room temperature from 200-250 nm with a scan rate of 50nm/minute and are an average of five accumulations. Samples contained 90µg/ml protein in 25mM sodium phosphate, 100mM NaCl, and 25% glycerol, pH 8.0. Estimates of secondary structure were determined with CDPro using a 29-protein reference set (46). Thermal denaturation studies also were performed with 90µg/ml protein in a 5mm path length cell. Unfolding was monitored at 222 nm from 5-100^{o}C.

Electrophoretic Mobility Shift Assays (EMSAs)

A 105bp fragment of the *amiE* promoter that contains its predicted Fur box (49), a 233bp fragment of the *pfr* promoter that contains its 3 predicted Fur boxes (26), and a 142bp fragment of the *rpoB* promoter (14) that contains no predicted Fur box were PCR amplified from WT G27 genomic DNA using the primer pairs listed in Table 8. Each fragment was purified using Performa DTR Gel Filtration Cartridges (Edge Bio) with a 1.5 minute elution at 4k rpm, and 150ng of each promoter fragment was then end labeled with ^{32}P and cleaned as previously described (14, 31). 50µL of $MnCl_2$-binding buffer ($MnCl_2$-BB) was added to the *amiE* and *rpoB* products while 50µL of *apo*-binding buffer (*apo*-BB) was added to the *pfr* and *rpoB* products.

amiE EMSAs were performed using iron substitution conditions achieved through the use of $MnCl_2$. These experiments were conducted in a manner analogous to that of

the previously described *sodB* EMSAs (14) with the following changes: 2x $MnCl_2$-BB (20% glycerol, 30mM Tris, pH 8.0, 120mM KCl, 16mM DTT, 480µg/mL BSA, 1mM $MnCl_2$, 0.03mg/mL sheared salmon sperm DNA) (31) was utilized in place of 2x *apo*-BB, Fur concentrations were 0.04µg/mL, 0.02µg/mL, and 0.01µg/mL, 500ng of unlabeled *amiE* DNA was used in the competition reactions, the 5% polyacrylamide gels were composed of 5% 19:1 acrylamide, 1x Tris Glycine (TG) buffer, 2.5% glycerol, and 0.133mM $MnCl_2$, and the gels were run in 1xTG buffer. Binding reactions were conducted using 1ng of labeled promoter with each of the Fur mutant proteins as well as WT Fur, and *rpoB* reactions were conducted under the same conditions to serve as the negative control. Samples were electrophoresed at 70V for three hours, gels were exposed to phosphor screens, and screens were scanned and analyzed as described above for the RPA experiments.

pfr EMSAs were performed under iron-free (*apo*) conditions as previously described for *sodB* (14, 29). The *rpoB* promoter was used as a negative control since Fur does not regulate expression of this gene. 10µL of 2x *apo*-BB (24% glycerol, 40mM Tris, pH 8.0, 150mM KCl, 2mM DTT, 600µg/mL BSA, 200µM EDTA, and 0.1mg/mL sheared salmon sperm DNA) was combined with 1ng of labeled promoter (either *pfr* or *rpoB*) and Fur at the following concentrations: 0.5µg/mL, 0.1µg/mL, and 0.02µg/mL. Additionally, a no protein control reaction was performed along with a 100ng cold (unlabeled) DNA competition. Reactions were incubated for 30 minutes at 37ºC and separated on a 5% polyacrylamide gel composed of 5% 19:1 acrylamide, 1x Tris Glycine EDTA (TGE) buffer, and 2.5% glycerol. The samples were electrophoresed in 1xTGE

buffer at 70V for three hours, the gels exposed to phosphor screens, and the screens scanned and analyzed as described above for the RPA experiments.

Iron Binding Studies

The ability of the purified WT Fur and the E90A, H96A, E110A, E117A, and H134A Fur mutant proteins to bind iron was determined by graphite furnace atomic absorption spectrophotometry following equilibrium dialysis. Briefly, 1ml of each protein (0.7 to 2.5μM) was dialyzed for at least 24 h at 4°C in a polyethylene-sealed Erlenmeyer flask against 1 liter of anoxic buffer that contained 50mM ultra-pure sodium chloride (Sigma), 10mM ultrapure sodium formate (Sigma), pH 7.5, Oxyrase (OB00-50) and increasing concentrations (0 to 12.5μM) of ultra-pure $FeCl_2$, 4 H_2O (Alfa Aesar). Oxyrase is a commercial blend of membrane enzymes used to scavenge oxygen from the medium. To ensure proper anaerobiasis, this buffer was left for at least 2 hours at room temperature before beginning dialysis at 4°C. The efficiency of this treatment was assessed after dialysis by checking the oxygen concentration with a Clarke-type oxygen electrode (30): no oxygen was detected in any of the dialysis baths. The pH of the dialysis buffer was checked before and after dialysis and was found to be 6.9 ± 0.1. In addition, the absence of protein contamination (by Oxyrase) in dialysis bags was verified by dialyzing a bag containing no Fur protein against the Oxyrase-containing buffer and by determining the protein concentration in the bag; the protein concentration was negligible. The iron concentration in the dialysis bag ("protein-bound plus free Fe^{2+}") and in the dialysis buffer ("free Fe^{2+}") was measured by atomic absorption using a Shimadzu AA-6701F spectrophotometer, and the concentration of protein-bound iron was estimated

by subtracting the two values. The number of iron molecules bound per monomer was then determined by dividing the concentration of protein-bound iron by the concentration of the protein alone as determined by BCA protein assay kit (Pierce Thermo Fisher) prior to the dialysis bath. All samples were diluted (in 0.1% HNO_3) to be in the range of the standard curve (0 to 0.4μM Fe) generated using an atomic absorption grade Fe standard solution (Sigma). Measurements were repeated for each sample until three replicates gave a coefficient of variation (CV) equal to or less than 12%. Results shown are the mean and standard deviation of 3 to 6 measurements.

Cross Linking Studies

To determine the ability of the Fur mutant proteins to form higher order structures, *in vitro* cross linking assays were performed similar to those previously described (24). Two μg of each individual protein was combined with 10uL of 1xPBS and 2μL of 25mM disuccinimidyl suberate (DSS) (Sigma) and allowed to incubate for one hour at room temperature. In addition, no DSS control reactions were set-up as described above without the cross linking reagent and each protein. After incubation, 5μL of 5x Laemmli sample buffer was added to each reaction, and the samples were boiled for 5 minutes at 95ºC. Samples were separated on SDS-PAGE gels, and Western Blot analysis of the cross linking reactions was performed as described above. For those Fur mutant proteins that showed a defect in their ability to form higher order structures, cross linking reactions were also performed using 1xPBS containing 1mM, 2mM, or 4mM $MnCl_2$ to see if the addition of excess Mn^{2+} (as an iron substitute) could restore the phenotype to that of the WT. In these cases, 2μg of each protein was incubated with the

respective $MnCl_2$ solution for two hours at room temperature prior to the addition of DSS and analysis.

Protein Modeling

H. pylori Fur was modeled after the *V. cholerae* dimeric structure as submitted to the NCBI's protein database under code 2W57 (44) using ESyPred3D (36).

Results

Comparison of Conserved Fur Residues and Selection of Mutant Targets

Given the fact that Fur has been well studied in *E. coli*, *P. aeruginosa*, and *V. cholerae* (4, 7, 19, 32, 35, 40, 42, 43) and due to the fact that the crystal structure from *P. aeruginosa* (42) was available when this study was initiated, we performed an amino acid alignment (37) of *H. pylori* Fur to Fur from these model organisms (Fig. 14). Compared to *E. coli*, *P. aeruginosa*, and *V. cholerae*, *H. pylori* Fur shares 34%, 28%, and 32% identity and 56%, 59%, and 55% similarity, respectively (11). Based on this alignment, there are 30 amino acid residues that are completely conserved among all four species. Nine of the conserved amino acid residues were found to be important for Fur functioning in at least one of the other organisms, and/or were predicted metal binding residues based on the *P. aeruginosa* crystal structure. Out of those nine the following residues were selected for mutation: V64, E90, H96, E110, E117, and H134.

Figure 14. Alignment of Fur amino acid Sequences.

The amino acid sequences of Fur from *H. pylori* were compared to that of *E. coli*, *V. cholerae*, and *P. aeruginosa* using ClustalW2 (37). Stars indicate completely conserved residues while periods (.) and colons (:) represent conserved substitutions and semi-conserved substitutions, respectively. Open circles, solid circles, and squares represent amino acid residues that have been shown to be important for Fur functioning in *E. coli*, *P. aeruginosa*, and *V. cholerae*, respectively. Triangles represent metal binding residues in the *P. aeruginosa* Fur crystal structure. Solid semi-circles indicate site specific amino acid mutations constructed in *H. pylori*.

Figure 14. Alignment of Fur amino acid Sequences.

Analysis of –iron-bound, -apo*, and –autoregulation*

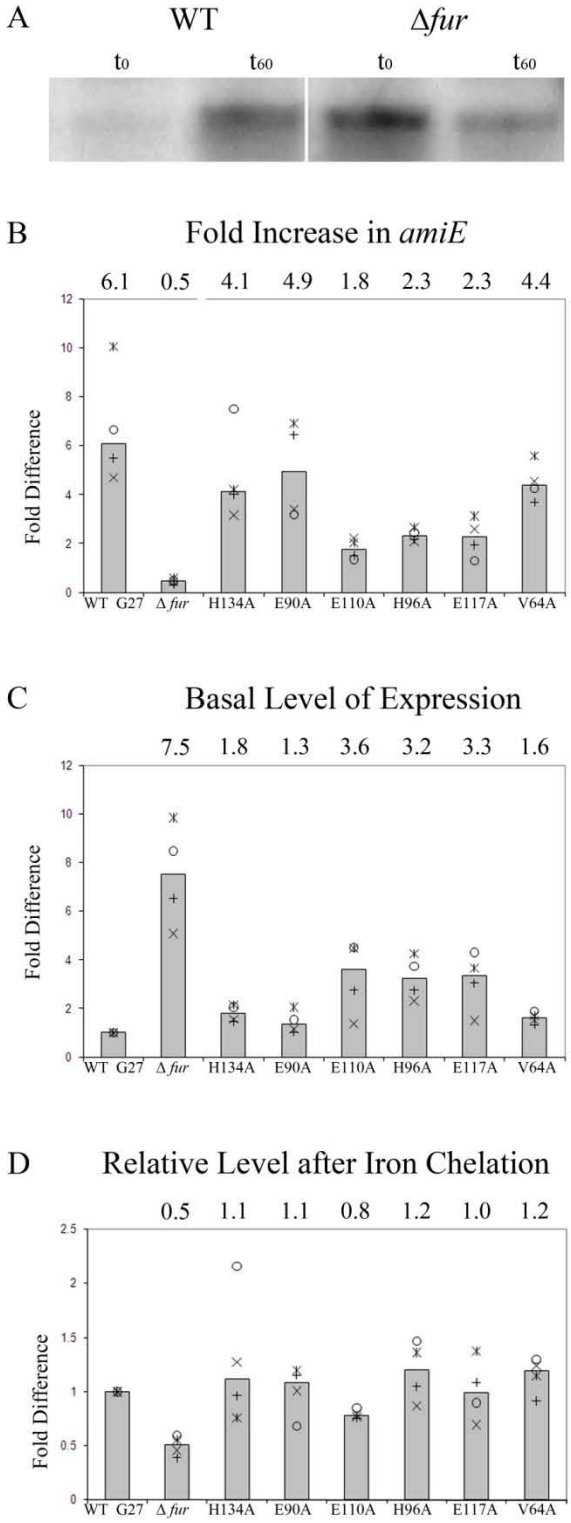
To determine whether alteration of the conserved amino acid residues affected the ability of *H.* pylori Fur to exhibit proper iron-bound regulation, we monitored Fur-dependent expression of the iron-repressed gene *amiE* (15, 49) in *H. pylori* strains that carried either a WT copy of Fur or one of the Fur mutations. *amiE* expression was monitored in iron replete media as compared to cells that had been exposed to iron starvation by an iron chelation shock. The fold change in expression was then calculated by comparing the amount of transcript present after iron chelation to the iron replete condition. Four biological repeats were conducted for the WT and each mutant strain, and the fold change in expression for each was plotted as a single point. The median fold change of the replicates for each strain (WT and mutant) is displayed as a bar (Fig. 15). As shown in Fig. 15A and 15B, iron chelation results in increased expression in *amiE* (6.1-fold), and this change in expression is completely lost in the Δ*fur* strain (0.5-fold). Three of the mutants exhibited changes in *amiE* expression similar to WT, H134A (4.1-fold), E90A (4.9-fold), and V64A (4.4-fold). Conversely, the other three mutants showed iron-bound regulatory affects on *amiE* expression. The E110A mutant displayed the smallest change in *amiE* expression (1.8-fold), while H96A and E117A exhibited intermediate changes (2.3-fold for each).

Given that the Δ*fur* mutant displays an increased basal level expression of *amiE* as compared to WT in the presence of iron (Fig. 15A and15C), we reasoned that site specific mutations that truly affect iron dependent regulation of Fur should show a similar phenotype. As shown in Fig. 15C, the E110A, H96A, and E117A mutants display an increase in basal level *amiE* expression (3.6, 3.2, and 3.3-fold, respectively). However,

Figure 15. Iron-bound Fur regulation of amiE *in the presence of various mutant Fur proteins.*

RNA was isolated from exponential growth phase *fur* mutant strains of *H. pylori* along with the WT and Δ*fur* controls under iron replete and iron-depleted shock conditions as detailed in the Materials and Methods. An *amiE* riboprobe was then used to assess the impact of the *fur* mutations on iron-bound Fur regulation by RPA. An example of an *amiE* RPA for WT and Δ*fur* under iron replete (t_0) and iron chelation shock conditions (t_{60}) is shown in panel A. The fold increase in *amiE* expression after iron chelation is shown in Panel B. The basal levels of *amiE* expression in iron replete conditions are shown in Panel C, and the relative levels of *amiE* expression after iron chelation shock are shown in Panel D. The fold increase and relative levels of expression are shown as single points for each strain with each shape (+, *, ○, and ×) representing one biologically independent replicate. The median fold increase and relative levels of expression are represented by the gray bars with the median numerical value indicated above the bars. The + represents the average value of two technical repeats for this biological replicate only.

Figure 15. Iron-bound Fur regulation of amiE *in the presence of various mutant Fur proteins.*



V64A, E90A, and H134A exhibited basal levels of expression akin to WT (1.6, 1.3, and 1.8-fold, respectively). Finally, we assessed the maximum level of expression displayed for each strain after iron chelation in comparison to the WT. As shown in Fig. 15D, all strains displayed similar levels of *amiE* expression post chelation. Taken together, these data indicate that the E110, E117, and H96 residues are important for iron-bound Fur regulation and suggest that these residues are involved in DNA binding, iron binding, and/or the ability of Fur to form higher order structures.

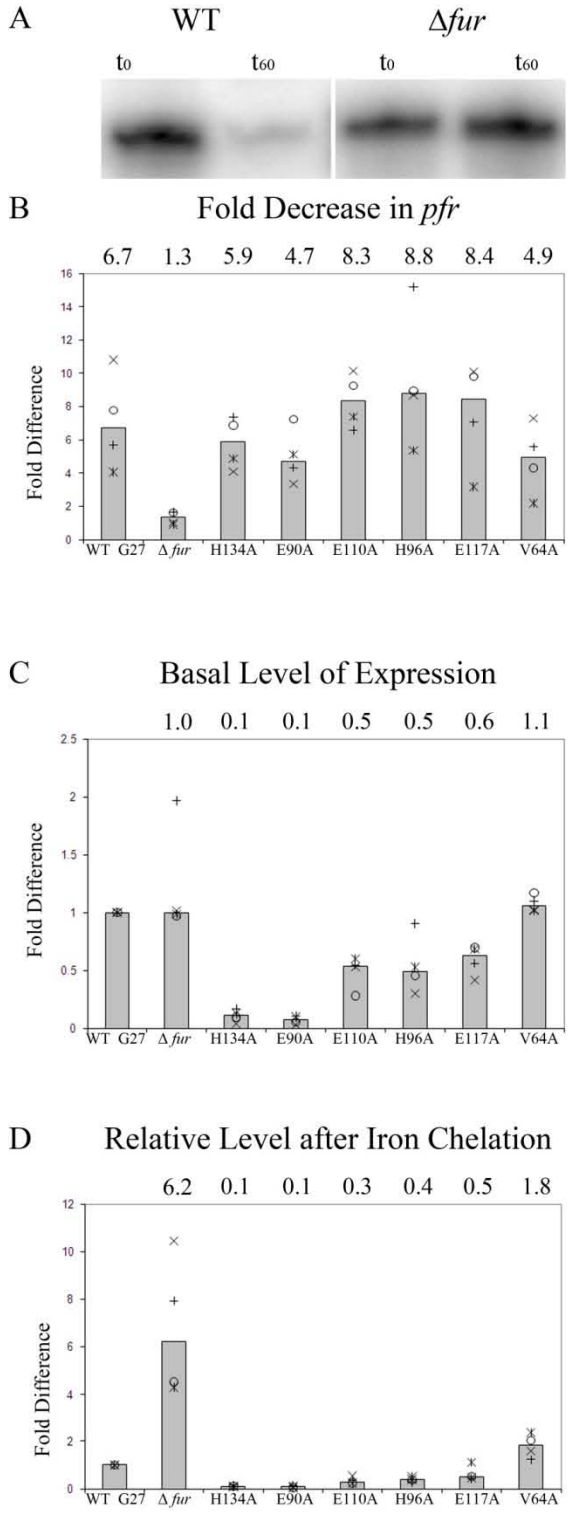
We next examined expression of *pfr*, which is repressed by *apo*-Fur (26), to determine if changes in any of the conserved amino acid residues affected the ability of *H. pylori* Fur to exhibit proper *apo* regulation. *pfr* expression was monitored under iron replete conditions as compared to cells that had been exposed to iron starvation through chelation. As with the *amiE* RPA analysis, fold change in *pfr* expression was calculated by comparing the level of expression after iron chelation shock to the level of expression under iron replete conditions. These data are displayed as described above (Fig. 16). As shown in Fig. 16A and 16B, iron chelation resulted in decreased expression of *pfr* (6.7-fold) and this decrease was lost in the Δ*fur* mutant (1.3-fold). Each of the Fur mutants exhibited decreases in *pfr* expression similar to WT (4.9, 4.7, 8.8, 8.3, 8.4, and 5.9-fold for V64A, E90A, H96A, E110A, E117A, and H134A, respectively).

Because there was no change in the basal level of *pfr* expression in the presence of iron in the absence of *fur* (Fig. 16A and 16C) and none of the mutants altered *pfr* regulation (Fig. 16B), we reasoned that the basal level of *pfr* expression in each of the mutant strains should be similar to WT. However, only one of the mutants, V64A, showed expression levels identical to WT (1.1-fold). H96A, E110A, and E117A

Figure 16. apo-*Fur regulation of* pfr *in the presence of various Fur mutant proteins.*

RNA was isolated from exponential growth phase *fur* mutant strains of *H. pylori* along with the WT and Δ*fur* controls under iron replete and iron-depleted shock conditions as detailed in the Materials and Methods. A *pfr* riboprobe was then used to assess the impact of the *fur* mutations on *apo*-Fur regulation by RPA. An example of a *pfr* RPA for WT and Δ*fur* under iron replete (t_0) and iron chelation shock conditions (t_{60}) is shown in panel A. The fold decrease in *pfr* expression after iron chelation is shown in Panel B. The basal levels of *pfr* expression in iron replete conditions are shown in Panel C, and the relative levels of *pfr* expression after iron chelation shock are shown in Panel D. The fold decrease and relative levels of expression are shown as single points for each strain with each shape (+, *, ○, and ×) representing one biologically independent replicate. The median fold increase and relative levels of expression are represented by the gray bars with the median numerical value indicated above the bars. The + represents the average value of two technical repeats for this biological replicate only.

Figure 16. apo-*Fur regulation of* pfr *in the presence of various Fur mutant proteins.*



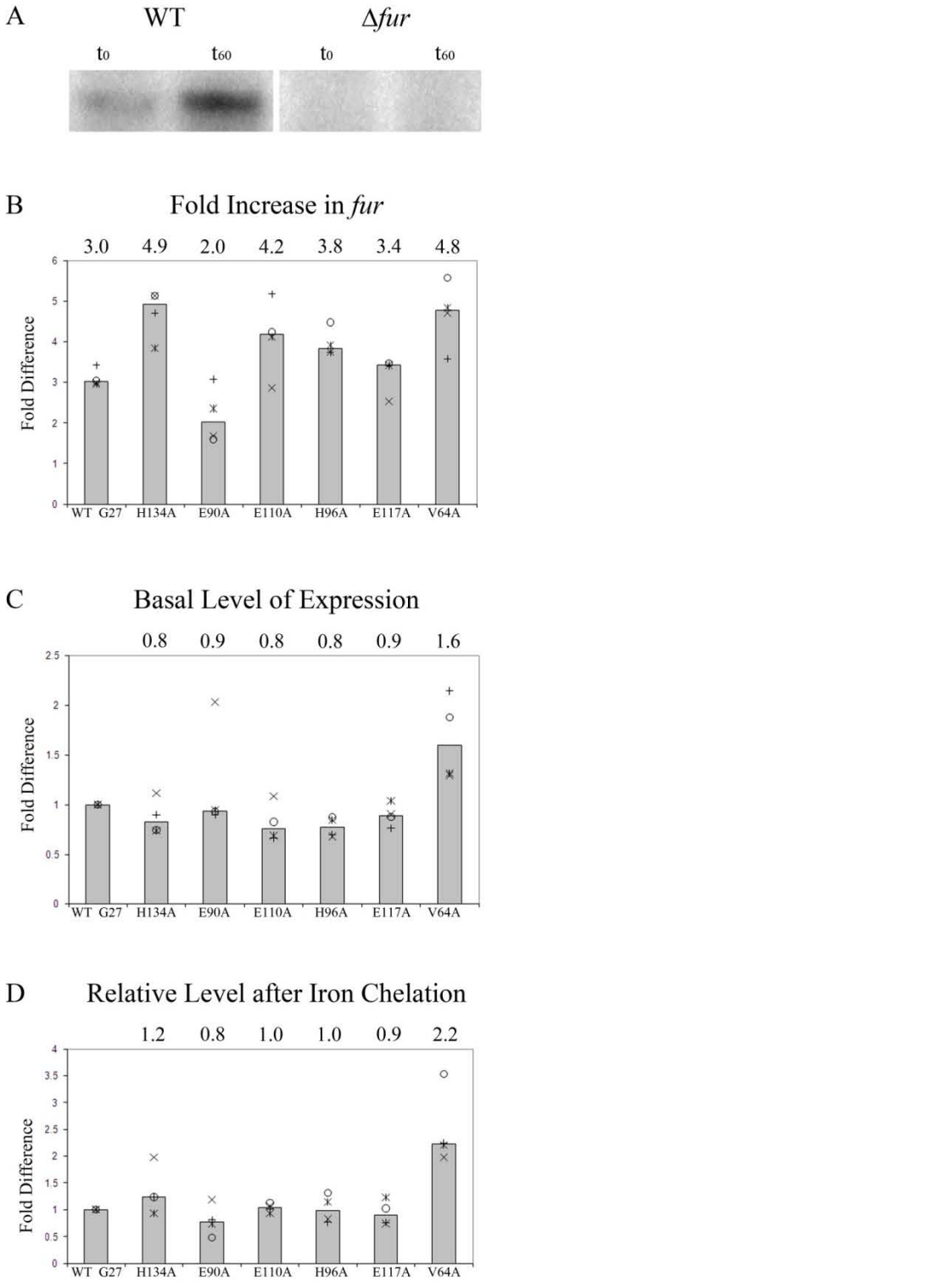
exhibited slight decreases in basal level *pfr* expression (0.5, 0.5, and 0.6-fold, respectively) while E90A and H143A displayed drastic decreases in the basal level of *pfr* (0.1-fold each, Fig. 16C). Next we analyzed *pfr* expression in each strain after iron chelation shock. As shown in Fig. 16D, in the absence of *fur*, *pfr* expression is 6.2-fold higher than in WT. The V64A strain expressed *pfr* similar to WT (1.8-fold) after chelation. The mutants, which had minor alterations in basal *pfr* expression (H96A, E110A, and E117A), also displayed minor alterations in expression in the absence of iron (0.4, 0.3, and 0.5-fold, respectively). E90A and H134A, which showed drastic reductions in basal level *pfr* expression, also showed drastic reductions in *pfr* expression as compared to WT after iron chelation (0.1-fold each). Taken together, these data suggest that while H96A, E110A, and E117A have a moderate affect on *apo* regulation, of the examined amino acid residues E90 and H134 appear most important for *apo*-Fur repression. Additionally, alteration of these amino acids results in a hyper-repressed phenotype in the presence and absence of iron, implying that these residues are important in DNA binding, iron binding, and/or in the formation of higher order structures.

Finally, to determine if any of the mutated amino acids residues were important for *fur* autoregulation, we monitored expression of *fur* (24, 25) in each strain grown in iron replete and iron deplete conditions. Upon removal of iron, *fur* expression is increased in WT bacteria (3.0-fold) as shown in Fig. 17A and 17B. Likewise, *fur* expression was increased in each of the mutants in a manner similar to WT (2.0-4.9-fold; Fig. 17B). Similarly, as shown in Fig. 17C, none of the mutants exhibited dramatic alterations in *fur* basal level expression (0.8-1.6-fold). Furthermore, *fur* expression post iron depletion remained virtually unchanged in the Fur mutant strains as compared to WT

Figure 17. fur *autoregulation in the presence of various mutant Fur proteins*.

RNA was isolated from exponential growth phase *fur* mutant strains of *H. pylori* along with the WT and Δ*fur* controls under iron replete and iron-depleted shock conditions as detailed in the Materials and Methods section. A *fur* riboprobe was then used to assess the impact of the *fur* mutations on Fur autoregulation by RPA. An example of a *fur* RPA for WT and Δ*fur* under iron replete (t_0) and iron chelation shock conditions (t_{60}) is shown in panel A. The fold increase in *fur* expression after iron chelation is shown in Panel B. The basal levels of *fur* expression in iron replete conditions are shown in Panel C, and the relative levels of fur expression after iron chelation shock are shown in Panel D. The fold increase and relative levels of expression are shown as single points for each strain with each shape (+, *, ○, and ×) representing one biologically independent replicate. The median fold increase and relative levels of expression are represented by the gray bars with the median numerical value indicated above each bar.

Figure 17. fur *autoregulation in the presence of various mutant Fur proteins.*



(0.8-2.2-fold; Fig. 17D). Together, these data indicate that none of the mutations significantly impact *fur* autoregulation.

En masse, these data indicate that the six mutations that we made to the protein sequence can be divided into the following classes: 1. Those with no effect (V64A), 2. Those that affect iron-bound Fur regulation (H96A, E110A, and E117A), and 3. Those that affect *apo*-Fur regulation (E90A and H134A).

Protein Accumulation and Structure

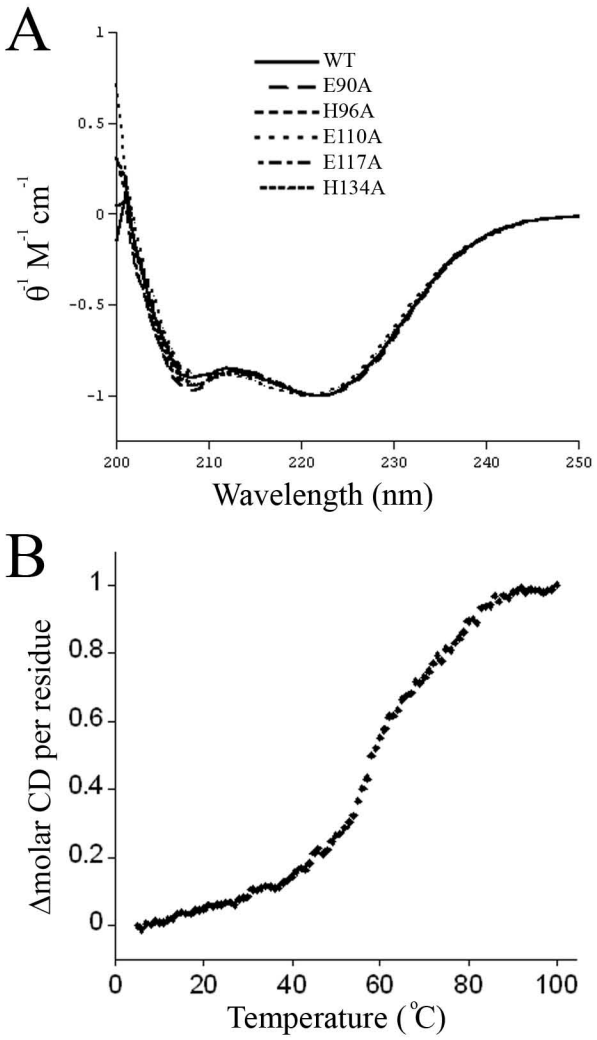
Given that five of the six mutant proteins showed a regulatory effect, we next analyzed protein accumulation and structure of these five proteins to ensure that the observed transcriptional changes were not due to gross changes in the relative level or structure of the Fur mutant. Western blot analysis revealed that each of the mutant Fur proteins was expressed and that there were no dramatic differences in the levels of each as compared to the WT (data not shown).

Circular dichroism (CD) studies on purified WT Fur and each of the five mutant proteins were conducted. The spectra of all of the proteins showed ordered secondary structure (Fig. 18A). The similarities between the spectra suggest that the proteins are composed of the same overall secondary structural features. Further analysis of the secondary structure by CDPro (46) showed that each of the Fur proteins contains on average 81.0 ± 0.3% helix, 5.3 ± 0.1 % turn and have 14.6 ± 0.1% unordered regions, which is in agreement with previous structural data from Fur in other bacterial species (42-44). Thermal denaturation experiments spanning 5-95°C for the mutant proteins resulted in profiles that are virtually indistinguishable from that of the WT protein (Fig.

Figure 18. Analysis of overall secondary structure in Fur mutant proteins.

CD spectra containing 5μM of each mutant Fur protein or WT Fur in 25mM sodium phosphate, 100mM NaCl and 25% glycerol at pH 8.0 were acquired at room temperature from 200-250nm at a rate of 50nm/minute for an average of five accumulations. The average normalized spectra for each protein are shown in Panel A as indicated in the inset. Thermal denaturation studies were also performed at 222nm on samples containing 5μM of each mutant Fur protein or WT Fur from 5-100ºC. Panel B shows a representative thermal denaturation profile of WT Fur.

Figure 18. Analysis of overall secondary structure in Fur mutant proteins.



18B and data not shown). The profiles show a single unfolding transition. Taken together, these data suggest that the observed alterations in iron-bound and *apo*-Fur regulation are not due to changes in Fur expression levels or to structural changes.

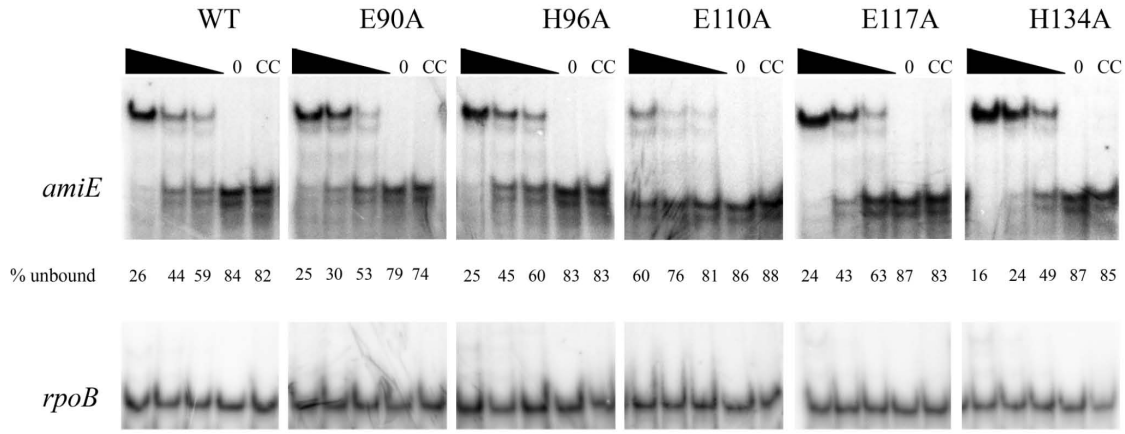
DNA Binding Studies

Given that no gross changes in protein accumulation or structure were identified, we next reasoned that changes in Fur regulation could be due to 1.) effects on DNA binding, 2.) effects on iron binding, and/or 3.) effects on the ability of Fur to form dimers or other higher order structures. We therefore sequentially checked each of these possibilities. To determine if any of the amino acid changes affected DNA binding, EMSAs were performed on the E90A, H96A, E110A, E117A, and H134A purified mutant Fur proteins as well as WT *H. pylori* Fur. First, to test iron-dependent binding, EMSAs were performed using the *amiE* Fur box region and each of the Fur mutant proteins. As shown in Fig. 19, WT Fur bound to the *amiE* fragment and altered the migration of the DNA even at the lowest concentration of Fur used. Furthermore, as the amount of Fur increased, the amount of unbound *amiE* DNA decreased. This interaction was specific as unlabeled *amiE* was able to compete for binding to Fur and "chase" the migration of the labeled fragment back to the location of the no protein control. Additionally, no shift in the migration of the control *rpoB* promoter fragment was observed under these conditions for any of the Fur proteins. Three of the five mutant proteins, E90A, H96A, and E117A, bound to the *amiE* DNA in a manner similar to WT while two, E110A and H134A, did not (Fig. 19). E110A exhibited a decreased ability to bind to the *amiE* promoter as evidenced by the increased amounts of unbound DNA

Figure 19. Analysis of iron-dependent DNA binding to the amiE *promoter.*

EMSAs were performed using purified WT or Fur mutant proteins (as indicated above each set) and an end-labeled *amiE* PCR fragment that encompasses the Fur box region. Labeled *rpoB* promoter fragment was used as the negative control for each protein. Increasing concentrations of protein (0.01μg/mL, 0.02μg/mL, and 0.04μg/mL) are indicated by the black triangles, the no protein control reactions are indicated by a "0", and the cold (unlabeled) competition reactions are indicated by the "CC". EMSAs shown are representative of 2-4 experimental repeats each performed with newly labeled *amiE* and *rpoB* fragments. The percent of unbound labeled *amiE* fragments is indicated below each reaction lane and is the median of the replicates.

Figure 19. Analysis of iron-dependent DNA binding to the amiE *promoter.*



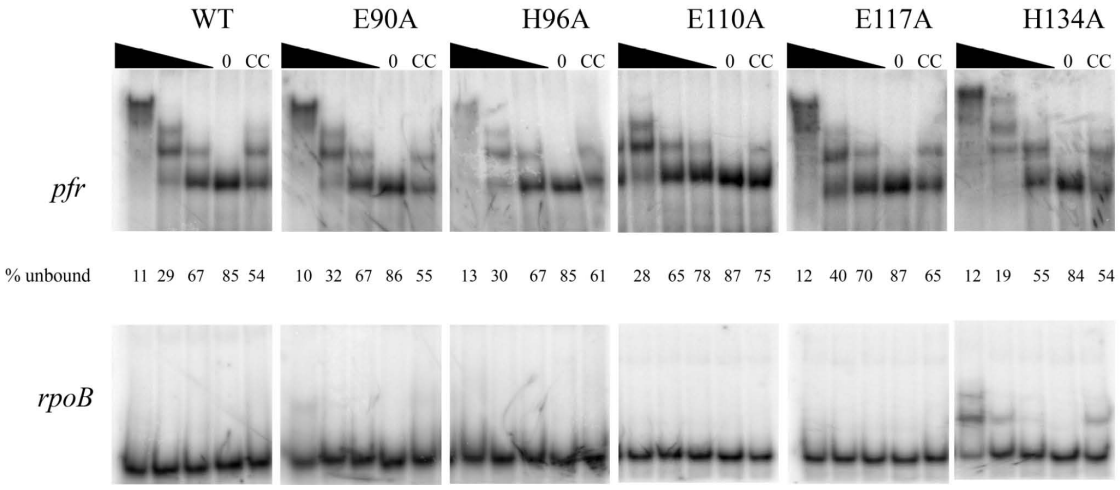
present at each of the protein concentrations. In contrast, H134A bound to the *amiE* promoter better than WT Fur; the percent of unbound *amiE* is considerably less at each protein concentration for H134A as compared to WT. Collectively, these data suggest that the E110 and H134 amino acid residues are important for Fur binding to target iron-bound promoter DNA while E90, H96, and E117 are not crucial for this aspect of Fur function.

To test if any of the amino acid mutations affected *apo*-dependent binding, EMSAs were performed using the Fur box region of *pfr*. *apo* conditions were achieved through the addition of EDTA, to chelate available iron from the system (14). As shown in Fig. 20, WT Fur bound to the *pfr* fragment and slowed its migration even at the lowest concentration of protein used. As the amount of Fur increased, *pfr* was shifted in a stepwise manner; one step for each of the three predicted Fur boxes (26) was evident. Competition with unlabeled *pfr* resulted in a shift in migration back towards the no protein control, and eliminated the upper two migrating bands. E90A, H96A, and E117A all interacted with *pfr* in a manner similar to WT while E110A and H134A exhibited alterations in *pfr* binding (Fig. 20). As seen in the iron-bound Fur EMSAs, E110A bound *pfr* less well than WT as indicated by the increased amount of unbound *pfr* at each concentration of protein. Also, similar to what was seen with iron-bound interaction with *amiE*, H134A bound to the *pfr* promoter better than WT as shown by the presence of less unbound *pfr* at the lowest two concentrations of protein. In addition, H134A also now bound the *rpoB* negative control. Binding to this control was not seen with any of the other Fur proteins. In fact, H134A also was able to bind to a portion of the *flaA* coding sequence, which was previously used a negative control (31) (data not shown). These

Figure 20. Analysis of apo-*dependent DNA binding to the* pfr *promoter.*

EMSAs were performed using purified WT and Fur mutant proteins (as indicated above each set) and an end-labeled *pfr* PCR fragment that encompasses the three Fur box predicted for this promoter. Labeled *rpoB* promoter fragment was used as the negative control for each protein. Increasing concentrations of protein (0.02μg/mL, 0.1μg/mL, and 0.5μg/mL) are indicated by the black triangles, the no protein control reactions are indicated by a "0", and the cold (unlabeled) competition reactions are indicated by the "CC". EMSAs shown are representative of 2-3 experimental repeats each performed with newly labeled *pfr* and *rpoB* fragments. The percent of unbound labeled *pfr* fragment is indicated below each reaction lane and is the median of the replicates.

Figure 20. Analysis of apo-*dependent DNA binding to the* pfr *promoter.*



data suggest that the H134A mutation leads to indiscriminant DNA binding. In all, these EMSA data suggest that the E110 and H134 residues are important for iron-bound and apo-Fur DNA binding and that the E90, H96, and E117 residues are not critical for the DNA binding capability of Fur.

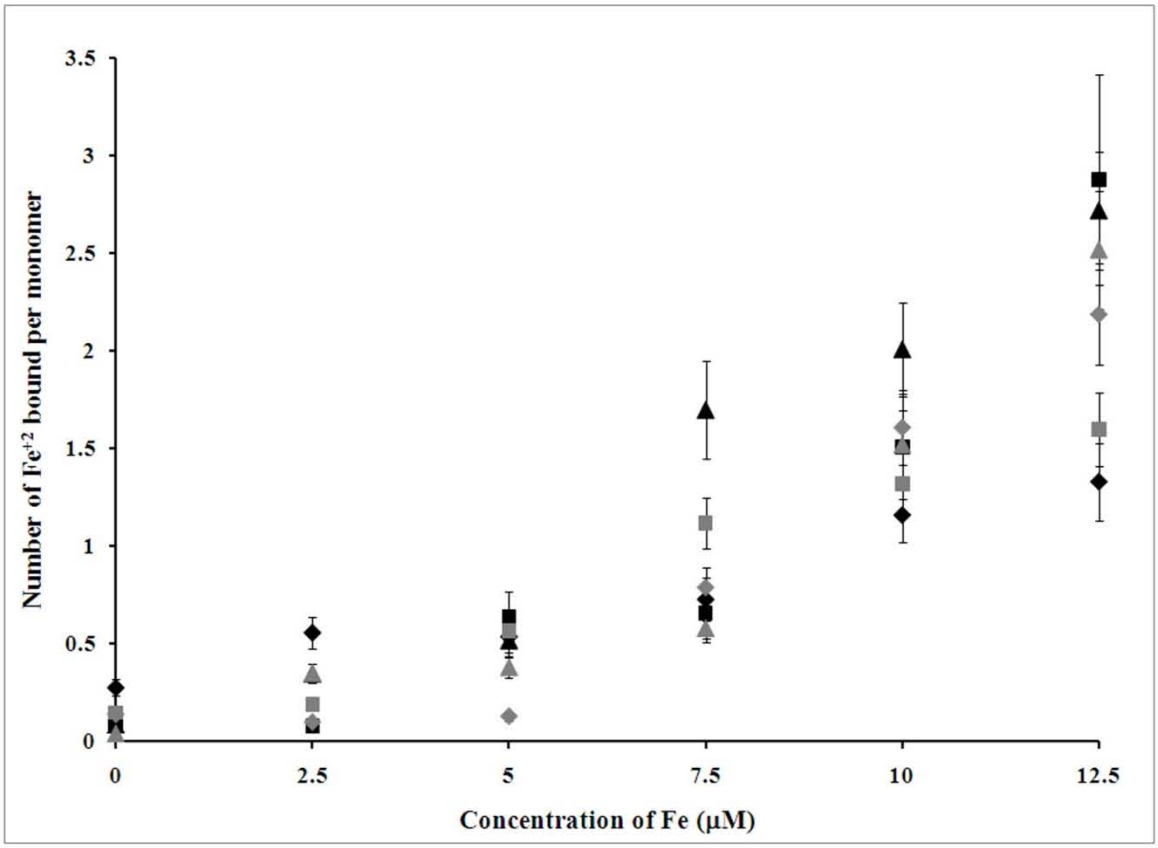
Iron Binding Studies

The second aspect of Fur function that we next examined for each of the Fur mutant proteins was the ability of each to bind iron. To determine the impact of the individual site specific mutations on the ability to bind iron, atomic absorption spectroscopy was performed on purified WT *H. pylori* Fur as well as the purified E90A, H96A, E110A, E117A, and H134A proteins that had been dialyzed in increasing concentrations of iron under anoxic conditions. As shown in Fig. 21, at the highest concentration of iron (12.5μM), WT Fur bound approximately 2-3 molecules of Fe^{2+} per monomer. Two of the five mutant Fur proteins, E90A and H134A, bound iron in a manner analogous to WT. Although E117A bound iron less well than WT, at the highest concentration of iron (12.5μM), E117A bound only slightly less iron; 2- 2.5 molecules per monomer were bound as compared to the 2-3 molecules per monomer for WT. H96A and E110A both bound iron less well than WT: 1-1.5 total molecules of Fe^{2+} were bound at the highest concentration of iron examined. Taken together, these data suggest that the E90 and H134 residues are not essential for the ability of Fur to bind to iron. Conversely, E117A plays a minor role in iron binding ability, and the H96 and E110 residues are critical for this aspect of Fur function.

Figure 21. Analysis of the ability of the Fur mutant proteins to bind iron.

Atomic absorption spectroscopy was performed on each purified Fur mutant protein, as well as WT Fur, following dialysis with increasing concentrations of Fe^{2+} (0.0 to 12.5μM). The number of Fe^{2+} molecules bound per monomer is indicated as follows for each protein: WT Fur (■), E90A (▲), H96A (◆), E110A (■), E117A (◆), and H134A (▲). Each point represents the mean of three to six replicates, and the standard deviation is given as bars for each point.

Figure 21. Analysis of the ability of the Fur mutant proteins to bind iron.



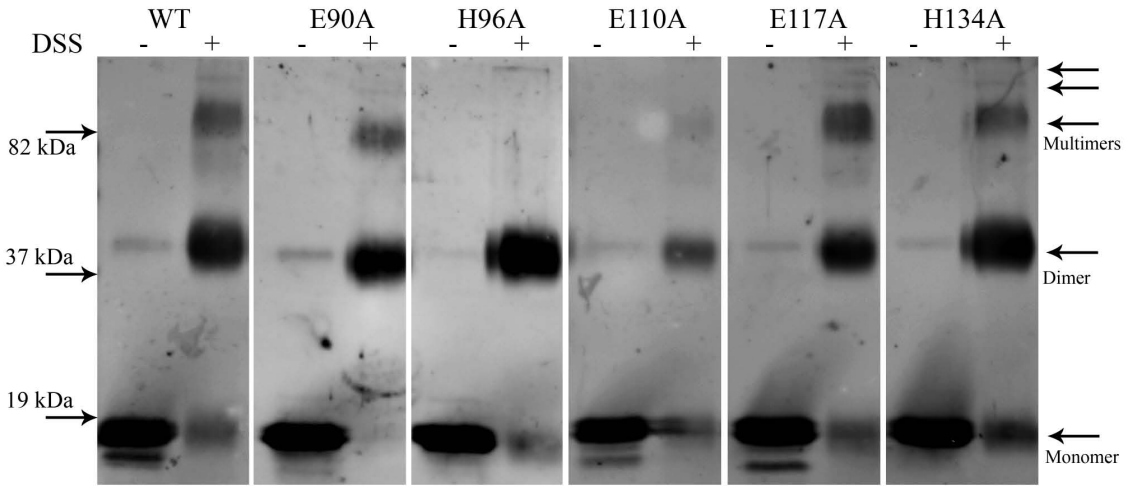
Formation of Higher Order Structures

Classic Fur regulation involves the formation of dimers and other higher order structures (1, 6, 42, 44); therefore, we next analyzed the ability of WT and mutant Fur proteins to oligomerize. Hence, *in vitro* cross linking studies were conducted with the cross linking reagent DSS. In the absence of DSS, the majority of the WT Fur protein was found in the monomeric form (approximately 17kDA), but a few stable dimers were also visible (Fig. 22). However, when the cross linking reagent was added, the amount of monomeric protein was significantly reduced and the dimeric and tetrameric forms of the protein predominated. Additionally, octameric and larger multimeric forms became visible (Fig. 22). The ability of Fur to form these higher order structures is in agreement with previous demonstrations of multimerization of WT *H. pylori* Fur (24). The E90A, E117A, and H134A Fur mutants all formed higher order structures in a manner similar to WT (Fig.22). Conversely, the H96A Fur mutant showed fewer dimers in the absence of DSS and no structures larger than dimers even in the presence of DSS (Fig. 22). Additionally, E110A showed a modest defect in the *in vitro* cross linking reactions; there were fewer dimers present in the absence of DSS and less dimers and tetramers present with DSS than in the WT control (Fig.22). Furthermore, the octomeric and multimeric forms were not clearly visible for this mutant. Taken together, these *in vitro* cross linking reactions suggest that the H96 and E110 amino acid residues are important for the formation of higher order structures.

Figure 22. Analysis of the dimerization ability of the Fur mutant proteins.

Cross linking studies were performed with 2μg of protein (WT, E90A, H96A, E110A, E117A, or H134A) incubated in 10μL 1xPBS in the presence (+) or absence (-) of 2μL of 25mM DSS for one hour at room temperature. Reactions were run on SDS-PAGE gels and protein was visualized via Western blot analysis with anti-Fur antibodies. Size markers and predicted higher order structures are indicated with arrows.

Figure 22. Analysis of the dimerization ability of the Fur mutant proteins.



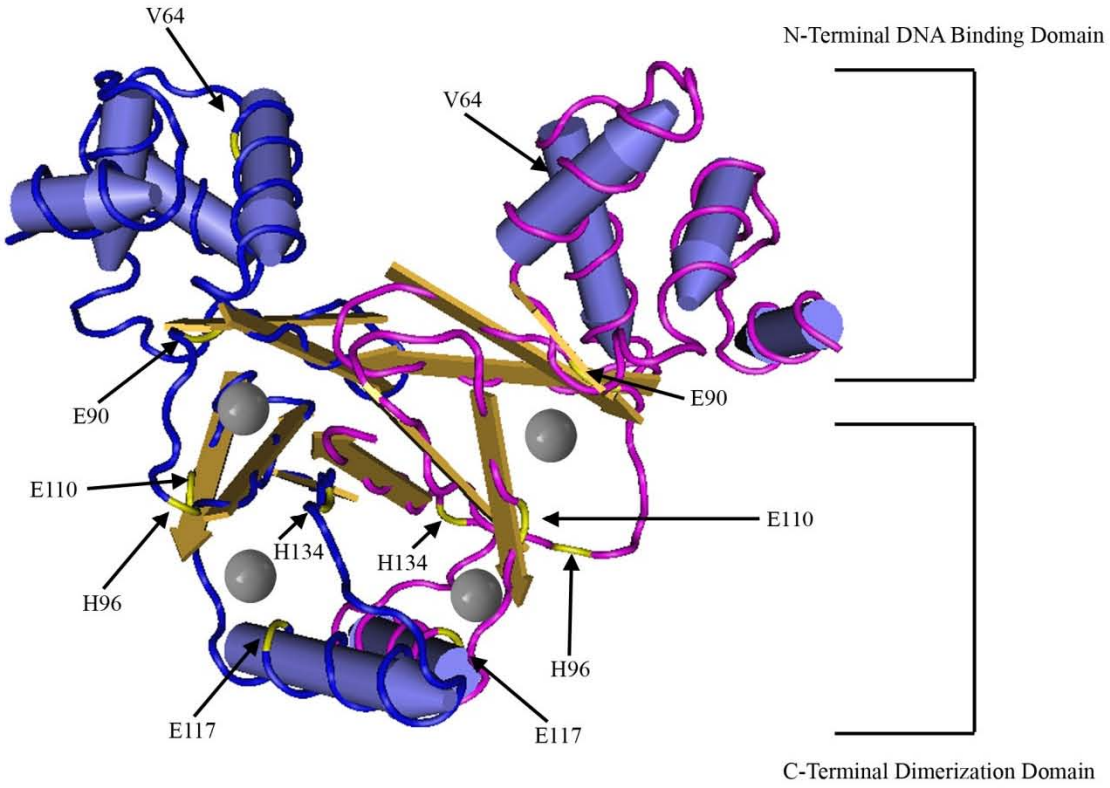
Discussion

Fur regulation in *H. pylori* is unique compared to other organisms in that Fur not only functions as a transcriptional repressor in the presence of its Fe^{2+} co-factor, but also has been shown to function as a repressor in its iron free, *apo* form. As yet, *apo*-Fur regulation has been characterized exclusively in *H. pylori* and thus, makes the study of Fur in this organism of particular interest. Fur amino acid sequences are highly conserved among bacteria (11, 22), and multiple broadly conserved residues have been shown to be important for Fur function across a diverse number of species. The bulk of this knowledge was garnered through mutagenesis studies and has been further supported by resolution of the *P. aeruginosa* and *V. cholerae* Fur crystal structures (42, 44). From these combined studies, it has been shown that the Fur monomer contains two domains: the N-terminal DNA binding domain and the C-terminal dimerization domain (20) (Fig 23). Within the C-terminal domain, there are two metal binding sites. The first, called site 1, is considered to be the regulatory site that mediates the necessary conformational change that allows Fur to bind to DNA and the second, called site 2, is considered to be the structural site that is necessary for dimerization of the two monomers (42). This naming scheme is based on the *P. aeruginosa* crystal structure (42). Among Fur proteins, the structural site has been found to bind either Fe^{2+} or Zn^{2+} depending on the species studied while Fe^{2+} is always bound at the regulatory site (39). Given that there is currently no *H. pylori* Fur crystal structure available, we took a genetic approach to determine what aspects of the structure/function of *H. pylori* Fur may facilitate the unique aspects of Fur regulation in this pathogen.

Figure 23. Model of H. pylori *Fur.*

A predicted 3D model of *H. pylori* Fur was generated based on the dimeric structure of *V. cholerae* Fur (44). One monomer is shown in magenta and the other in blue. Amino acid residues that were selected for mutation are shown in yellow and indicated by labeled arrows. The N-terminal DNA binding domain and the C-terminal dimerization domain are indicated. Metal ions are shown as gray spheres.

Figure 23. Model of H. pylori *Fur.*



In the studies described here, six amino acid mutations were constructed in broadly conserved residues (V64A, E90A, H96A, E110A, E117A, and H134A), which, as described below, have been shown to be important for Fur function in other species. A model of *H. pylori* Fur with the location of each of these residues indicated is shown in Fig. 23. Of the six mutations that were constructed, one (V64A) did not alter either iron-bound or *apo*-Fur regulation, two (E90A and H134A) altered *apo*-Fur regulation, and three (H96A, E110A, and E117A) altered iron-bound regulation (Fig. 15-17). Although H96A, E110A, and E117A did have a slight affect on *apo*-Fur regulation of *pfr* (Fig. 16), we have not classified them as altering both types of Fur regulation because their impact on *apo*-Fur repression was not as significant as that of E90A and H134A. This could be because the structure of *apo*-Fur is different from that of iron-bound Fur, and the E90 and H134 residues are more prominent in the *apo* form than are the other residues studied here. Given the importance of Fur in *H. pylori*, perhaps, it is not surprising that none of the constructed mutants displayed a Δ*fur* phenotype. This fact, as suggested in a recent review of the Fur family of metalloregulators (39), supports the notion that Fur is a robust protein that has evolved the capacity to maintain regulatory function even in the midst of some mutations.

Of the mutations that showed a regulatory phenotype, H96A was shown to result in decreased iron-binding and oligomerization abilities, E110A resulted in decreased iron-binding, DNA-binding and oligomerization abilities, E117A resulted in a slight iron-binding deficiency, H134A resulted in increased DNA binding, and E90A did not noticeably affect any of the specific mechanisms of Fur function that we tested. A summary of these results is shown in Table 9. Also of note, *fur* autoregulation remained

Table 9. Summary of Mutant Fur Protein Phenotypes[a]

Class	Iron-bound Fur Regulation[b]			*apo*-Fur Regulation[c]	
Mutation	H96A	E110A	E117A	E90A	H134A
amiE EMSA	WT	<WT	WT	WT	>WT
pfr EMSA	WT	<WT	WT	WT	>WT
Fe-binding	<WT	<WT	≤WT	WT	WT
Oligomerization	<WT	<WT	WT	WT	WT

[a] WT indicates levels similar to wild-type, > indicates levels greater than WT, < indicates levels less than WT, and ≤ indicated levels less than or equal to WT.

[b]Defined as *in vivo* effect on *amiE* in Fig. 2.

[c]Defined as *in vivo* effect on *pfr* in Fig. 3.

unchanged in the presence of each mutant Fur protein. This is likely the result of the combination of iron-bound Fur repression and *apo*-Fur activation that are required for the complex autoregulation of *fur* expression, which may have evolved to help maintain a constant level of *fur* expression within the cell (25).

Given that V64 is predicted to lie in the DNA binding domain of Fur and since mutation of the same residue in *V. cholerae* Fur does alter Fur regulation (35), the lack of a regulatory phenotype for the V64A mutation (Fig. 15-17) was somewhat surprising. The fact that V64 appears not to be required in *H. pylori* could indicate that despite the overall conservation between the proteins, the structures of Fur from these two organisms may differ within this region. This suggestion is perhaps supported by the alignment of Fur protein sequences (Fig. 14). The first 10 residues in *H. pylori* Fur do not align to any of the other Fur sequences used for direct comparison in this study, but similar N-terminal extensions are found in *Helicobacter hepaticus, Helicobacter acinonychis, Helicobacter cinaedi, Helicobacter bilis, Helicobacter canadensis, Helicobacter pullorum, Helicobacter winghamensis, Wolinella succinogenes,* and *C. jejuni* Fur sequences (data not shown), which, like *H. pylori*, are also members of the ε-Proteobacteria. The presence of this N-terminal extension only in these related species could indicate that the structure of Fur, or at least the DNA binding region, differs among this class of bacteria as a result of an evolutionary phenomenon. It is also interesting to note that analysis of the N-terminal DNA binding domain of *H. pylori* Fur (approximately the first 83 amino acids) shows that in comparison to the same region of *P. aeruginosa*, *H. pylori* Fur is much more basic (M. Vasil, personal communication). This likely changes the way *H. pylori* Fur and its closely related Fur proteins interact with

target DNA. Perhaps there is a role for this region in *apo*-Fur regulation as *C. jejuni* is also predicted to utilize Fur in this manner (33).

H96 lies in the highly conserved HHDH region that has long been predicted to be important for metal binding (4, 35, 43). Indeed, resolution of the *P. aeruginosa* Fur crystal structure (42) showed that H96 was involved in metal binding. Mutation of this residue in *H. pylori* resulted in decreased iron binding (Fig. 21) suggesting that it plays a similar role in *H. pylori*. Additionally, we found that the H96A mutant Fur protein exhibited a decreased ability to form higher order structures (Fig. 22). Given its defect in iron binding, we wondered whether the cross linking deficiency could be compensated for by the addition of excess Mn^{2+} to the buffers (to mimic increased Fe^{2+} substitution). Thus, cross linking studies using buffers containing increasing amounts of $MnCl_2$ showed that the addition of extra metal was able to restore WT levels of higher order structure formation to the H96A mutant protein (data not shown). This indicates that as with other organisms (43), the decreased ability to bind iron impacts the ability of *H. pylori* Fur to form higher order structures.

As mentioned above, the E110 residue is important for Fur regulation in *V. cholerae* (35) and is one of the predicted metal binding sites in *P. aeruginosa* Fur (42); therefore, it is not surprising that iron binding is altered in the *H. pylori* E110A Fur mutant protein (Fig 8), and this likely accounts for its slight dimerization defect (Fig. 22). Additionally, in *H. pylori*, this residue was important for proper binding to target DNA (Fig. 19) and for proper regulation of *amiE in vivo* (Fig. 15). In light of these roles, the observed alterations in *amiE* regulation seen in strains carrying this mutation make sense; if the E110A mutant Fur cannot bind iron and dimerize as well as WT and this results in a

diminished ability to bind to DNA, then we would expect to see increased basal levels of *amiE* expression and diminished changes in expression upon iron chelation (Fig. 15). What is perhaps more intriguing about this particular mutation is that the E110A protein also bound to the *pfr* Fur-box region with weaker affinity than WT under *apo* EMSA conditions (Fig. 20). However, *in vivo* the regulation of *pfr* was not affected by the slightly decreased affinity (Fig. 16). The overall change in DNA binding to *pfr* was less significant than for the *amiE* promoter. In general, the affinity of *apo*-Fur for *apo*-regulated promoters is significantly less than that of iron-bound Fur for the *amiE* promoter (29, 49); based on the decreased affinity for *apo*-regulated genes, significantly less Fur was required for use in the iron-bound EMSAs than was needed for the *apo* EMSAs. Given the decreased affinity of Fur for *apo* regulated genes (29), it may not be surprising that the E110A mutation did not affect *apo*-Fur regulation (Fig. 16); a slight further decrease in DNA binding ability may not have a noticeable impact on regulation. Conversely, a decrease in DNA binding to the high affinity *amiE* promoter (49) would be readily observed.

Based on the *P. aeruginosa* crystal structure, residues E90, E117 and H134A were also predicted to be important for iron binding. However, mutation of these residues in *H. pylori* Fur only slightly altered iron binding for the E117A mutant protein and not at all for the E90A and H134A mutant proteins. Because the E117A mutation only minimally impacted iron binding, it makes sense that *amiE* regulation in the strain carrying this mutation was only slightly altered in the presence of this mutation. On the other hand, the H134A mutant Fur protein exhibited increased affinity for DNA in both its iron-bound and *apo* forms, which we would not have predicted based on our

understanding of this residue's role in *P. aeruginosa* Fur. This suggests that in *H. pylori*, the H134 residue is involved in more than just iron binding. Perhaps alteration of this residue changes the tertiary structure of Fur such that the DNA binding domain is in a conformation that is more readily able to interact with DNA and thus "locks" Fur in its DNA binding conformation. In contrast to the other residues, mutation of E90 did not interfere with any of the tested aspects of Fur function despite the fact that the strain carrying the E90A mutation showed alteration of *apo*-Fur regulation of *pfr*. While unexpected, this finding perhaps suggests that alteration of this residue may result in slight modifications to Fur that our analyses were not sensitive enough to observe. Alternatively, the E90A mutation may impact some aspect of Fur function for which we did not assay or the results differ due to differences in *in vivo* vs. *in vitro* assays.

Considering the overall location of all of the constructed *H. pylori fur* mutations in this study as shown in Fig. 23, one (V64A) lies within the DNA-binding domain while the others (E90A, H96A, E110A, and E117A, and H134A) lie within one of the two metal binding sites suggested by the *P. aeruginosa* crystal structure (42). H96A, E117A, and H134A are located in regulatory site 1, and E90A and E110A are located in structural site 2. However, as discussed in a recent review by Lee and Helmann, the roles of sites 1 and 2 may not be as simple or specific as described for *P. aeruginosa* Fur (39). Adding to this, the analysis of the crystal structure of *V. cholerae* Fur (44) and model-based binding free-energy calculations (1) indicate that in fact the role of these sites may be reversed; this would mean that site 1 is really the structural site and site 2 is the regulatory site.

The analysis of our *H. pylori* Fur mutants adds more evidence to the complex and perhaps interdependent roles of the two metal binding sites. Of the mutated residues that lie in site 1, only H134A was found to actually impact DNA binding ability of the *H. pylori* protein; H96A affected the ability of the protein to bind iron and form higher order structures, which are roles typically associated with structural site 2 residues. In addition, E110A altered the DNA binding ability of Fur, but lies in the structural site (site 2), which is thought to be important for dimerization. Taken together, if the metal binding sites from *P. aeruginosa* are preserved in *H. pylori* Fur, it is apparent that the distinctions in functioning of sites 1 and 2 are blurred and likely work in a more coordinated manner.

It is also interesting to note that based on the data presented here, it does not appear that all of the predicted metal binding residues within sites 1 and 2 have an equal impact on Fur function. For instance, E90 and E110 both lie in site 2, yet mutation of the E110 residue impacted DNA binding, iron binding and oligomerization while mutation of E90 did not affect any of these aspects of Fur function. Also, the *in vivo* data suggests that E110 is important for iron-bound Fur regulation while E90 is important for *apo*-Fur regulation. Likewise, in site 1, mutation of E117 produced only minimal changes in iron binding while mutation of H96 and H134 resulted in pronounced changes in iron binding and oligomerization and DNA binding, respectively. In addition, one residue in each site altered *apo*-Fur regulation of *pfr in vivo* – H134 in site 1 and E90 in site 2. Similarly, the residues that impacted iron-bound Fur regulation of *amiE in vivo* were split between the two sites, H96 and E117 in site 1 and E110 in site 2. These data suggest that the metal binding residues do not have an equal functional role and that both metal binding site 1 and site 2 are important for iron-bound and *apo*-Fur regulation.

Further, adding to the apparent complexity of the Fur structure function relationship is the recent report that the structural site in *H. pylori* Fur is coordinated by a Zn^{2+} molecule (51) as is that of *P. aeruginosa* (42) and *E. coli* (3, 34). The Zn^{2+} molecule in *H. pylori* is coordinated by two CXXC motifs consisting of amino acid residues: C102, C105, C142, and C145 and has no resemblance to the structural Zn^{2+} site (site 2) of *P. aeruginosa* Fur (51), which lacks cysteines. This finding makes *H. pylori* Fur more like that of *E. coli*, where two C residues are implicated in metal binding (19), and PerR of *Bacillus subtilis* (a metalloprotein within the Fur family), which coordinates a structural Zn^{2+} molecule through four C residues (38, 48, 51). Perhaps, the ability of Fur to bind not only iron but also zinc at one or both of the metal binding sites provides a means of facilitating or adapting regulation of target genes as metal availability changes may slightly alter the dimerization or DNA binding abilities of the protein. Future mutational studies on *H. pylori* Fur should be directed at understanding the overall role of these two CXXC residues.

Taken together, these data continue to highlight the uniqueness of *H. pylori* Fur not only in terms of its regulation but in what is necessary for Fur to function. The use of Fur as a repressor in its *apo* form has not been characterized in other bacterial species, yet it is well documented in this organism (14, 15, 26, 29). We propose that the N-terminal extension in *H. pylori* Fur that is not seen in any of the model organisms in which Fur has been well characterized likely alters the interaction of *H. pylori* Fur with the target DNA and contributes to its unique regulatory capabilities. The residues predicted to be involved in metal binding appear to impact multiple aspects of Fur function for both iron-bound and *apo*-Fur regulation. Clearly, in the absence of a crystal structure, studies like

that of Vitale *et al*., (51) and the one presented here will continue to be essential for gaining insight into how Fur functions as well as the amino acid residues important for proper gene regulation in this medically important pathogen.

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References

1. **Ahmad, R., B. O. Brandsdal, I. Michaud-Soret, and N. P. Willassen.** 2009. Ferric uptake regulator protein: binding free energy calculations and per-residue free energy decomposition. Proteins **75:**373-86.

2. **Alamuri, P., N. Mehta, A. Burk, and R. J. Maier.** 2006. Regulation of the *Helicobacter pylori* Fe-S cluster synthesis protein NifS by iron, oxidative stress conditions, and Fur. J Bacteriol **188:**5325-30.

3. **Althaus, E. W., C. E. Outten, K. E. Olson, H. Cao, and T. V. O'Halloran.** 1999. The ferric uptake regulation (Fur) repressor is a zinc metalloprotein. Biochemistry **38:**6559-69.

4. **Arnold, F. H., and B. L. Haymore.** 1991. Engineered metal-binding proteins: purification to protein folding. Science **252:**1796-7.

5. **Bagg, A., and J. B. Neilands.** 1987. Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry **26:**5471-7.

6. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol Rev **51:**509-18.

7. **Barton, H. A., Z. Johnson, C. D. Cox, A. I. Vasil, and M. L. Vasil.** 1996. Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. Mol Microbiol **21:**1001-17.

8. **Bender, K. S., H. C. Yen, C. L. Hemme, Z. Yang, Z. He, Q. He, J. Zhou, K. H. Huang, E. J. Alm, T. C. Hazen, A. P. Arkin, and J. D. Wall.** 2007. Analysis of a ferric uptake regulator (Fur) mutant of *Desulfovibrio vulgaris* Hildenborough. Appl Environ Microbiol **73:**5389-400.

9. **Bereswill, S., S. Greiner, A. H. van Vliet, B. Waidner, F. Fassbinder, E. Schiltz, J. G. Kusters, and M. Kist.** 2000. Regulation of ferritin-mediated cytoplasmic iron storage by the ferric uptake regulator homolog (Fur) of *Helicobacter pylori*. J Bacteriol **182:**5948-53.

10. **Bereswill, S., F. Lichte, S. Greiner, B. Waidner, F. Fassbinder, and M. Kist.** 1999. The ferric uptake regulator (Fur) homologue of *Helicobacter pylori*: functional analysis of the coding gene and controlled production of the recombinant protein in *Escherichia coli*. Med Microbiol Immunol **188:**31-40.

11. **Bereswill, S., F. Lichte, T. Vey, F. Fassbinder, and M. Kist.** 1998. Cloning and characterization of the fur gene from Helicobacter pylori. FEMS Microbiol Lett **159:**193-200.

12. **Blaser, M. J.** 1990. Epidemiology and pathophysiology of *Campylobacter pylori* infections. Rev Infect Dis **12 Suppl 1:**S99-106.

13. **Bury-Mone, S., J. M. Thiberge, M. Contreras, A. Maitournam, A. Labigne, and H. De Reuse.** 2004. Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. Mol Microbiol **53:**623-38.

14. **Carpenter, B. M., H. Gancz, R. P. Gonzalez-Nieves, A. L. West, J. M. Whitmire, S. L. Michel, and D. S. Merrell.** 2009. A single nucleotide change affects fur-dependent regulation of *sodB* in *H. pylori*. PLoS One **4:**e5369.

15. **Carpenter, B. M., T. K. McDaniel, J. M. Whitmire, H. Gancz, S. Guidotti, S. Censini, and D. S. Merrell.** 2007. Expanding the *Helicobacter pylori* genetic toolbox: modification of an endogenous plasmid for use as a transcriptional reporter and complementation vector. Appl Environ Microbiol **73:**7506-14.

16. **Carpenter, B. M., J. M. Whitmire, and D. S. Merrell.** 2009. This is not your mother's repressor: the complex role of Fur in pathogenesis. Infect Immun **77:**2590-601.

17. **Chalker, A. F., H. W. Minehart, N. J. Hughes, K. K. Koretke, M. A. Lonetto, K. K. Brinkman, P. V. Warren, A. Lupas, M. J. Stanhope, J. R. Brown, and P. S. Hoffman.** 2001. Systematic identification of selective essential genes in *Helicobacter pylori* by genome prioritization and allelic replacement mutagenesis. J Bacteriol **183:**1259-68.

18. **Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, and et al.** 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A **90:**5791-5.

19. **Coy, M., C. Doyle, J. Besser, and J. B. Neilands.** 1994. Site-directed mutagenesis of the ferric uptake regulation gene of *Escherichia coli*. Biometals **7:**292-8.

20. **Coy, M., and J. B. Neilands.** 1991. Structural dynamics and functional domains of the fur protein. Biochemistry **30:**8201-10.

21. **Danielli, A., D. Roncarati, I. Delany, V. Chiarini, R. Rappuoli, and V. Scarlato.** 2006. In vivo dissection of the *Helicobacter pylori* Fur regulatory circuit by genome-wide location analysis. J Bacteriol **188:**4654-62.

22. **de Lorenzo, V., J. Perez-Martin, L. Escolar, G. Pesole, and G. Bertoni.** 2004. Mode of Binding of the Fur Protein to Target DNA: Negative Regulation of Iron-Controlled Gene Expression, p. 185-196. *In* J. H. Crosa, Mey, A.R.,Payne, S.M. (ed.), Iron Transport in Bacteria. ASM Press, Washington, DC.

23. **Delany, I., A. B. Pacheco, G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. Iron-dependent transcription of the *frpB* gene of *Helicobacter pylori* is controlled by the Fur repressor protein. J Bacteriol **183:**4932-7.

24. **Delany, I., G. Spohn, A. B. Pacheco, R. Ieva, C. Alaimo, R. Rappuoli, and V. Scarlato.** 2002. Autoregulation of *Helicobacter pylori* Fur revealed by functional analysis of the iron-binding site. Mol Microbiol **46:**1107-22.

25. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2003. An anti-repression Fur operator upstream of the promoter is required for iron-mediated transcriptional autoregulation in *Helicobacter pylori*. Mol Microbiol **50:**1329-38.

26. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. Mol Microbiol **42:**1297-309.

27. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin Microbiol Rev **10:**720-41.

28. **Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. van Vliet, and G. Homuth.** 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. Microbiology **151:**533-46.

29. **Ernst, F. D., G. Homuth, J. Stoof, U. Mader, B. Waidner, E. J. Kuipers, M. Kist, J. G. Kusters, S. Bereswill, and A. H. van Vliet.** 2005. Iron-responsive regulation of the *Helicobacter pylori* iron-cofactored superoxide dismutase SodB is mediated by Fur. J Bacteriol **187:**3687-92.

30. **Ferber, D. M., and R. J. Maier.** 1993. Hydrogen-ubiquinone oxidoreductase activity by the *Bradyrhizobium japonicum* membrane-bound hydrogenase. FEMS Microbiol Lett **110:**257-64.

31. **Gancz, H., S. Censini, and D. S. Merrell.** 2006. Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen *Helicobacter pylori*. Infect Immun **74:**602-14.

32. **Hassett, D. J., P. A. Sokol, M. L. Howell, J. F. Ma, H. T. Schweizer, U. Ochsner, and M. L. Vasil.** 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron

uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. J Bacteriol **178:**3996-4003.

33. **Holmes, K., F. Mulholland, B. M. Pearson, C. Pin, J. McNicholl-Kennedy, J. M. Ketley, and J. M. Wells.** 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology **151:**243-57.

34. **Jacquamet, L., D. Aberdam, A. Adrait, J. L. Hazemann, J. M. Latour, and I. Michaud-Soret.** 1998. X-ray absorption spectroscopy of a new zinc site in the Fur protein from *Escherichia coli*. Biochemistry **37:**2564-71.

35. **Lam, M. S., C. M. Litwin, P. A. Carroll, and S. B. Calderwood.** 1994. *Vibrio cholerae fur* mutations associated with loss of repressor activity: implications for the structural-functional relationships of Fur. J Bacteriol **176:**5108-15.

36. **Lambert, C., N. Leonard, X. De Bolle, and E. Depiereux.** 2002. ESyPred3D: Prediction of proteins 3D structures. Bioinformatics **18:**1250-6.

37. **Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins.** 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23:**2947-8.

38. **Lee, J. W., and J. D. Helmann.** 2006. Biochemical characterization of the structural Zn2+ site in the *Bacillus subtilis* peroxide sensor PerR. J Biol Chem **281:**23567-78.

39. **Lee, J. W., and J. D. Helmann.** 2007. Functional specialization within the Fur family of metalloregulators. Biometals **20:**485-99.

40. **Lewin, A. C., P. A. Doughty, L. Flegg, G. R. Moore, and S. Spiro.** 2002. The ferric uptake regulator of *Pseudomonas aeruginosa* has no essential cysteine residues and does not contain a structural zinc ion. Microbiology **148:**2449-56.

41. **Merrell, D. S., L. J. Thompson, C. C. Kim, H. Mitchell, L. S. Tompkins, A. Lee, and S. Falkow.** 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. Infect Immun **71:**6510-25.

42. **Pohl, E., J. C. Haller, A. Mijovilovich, W. Meyer-Klaucke, E. Garman, and M. L. Vasil.** 2003. Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. Mol Microbiol **47:**903-15.

43. **Saito, T., M. R. Wormald, and R. J. Williams.** 1991. Some structural features of the iron-uptake regulation protein. Eur J Biochem **197:**29-38.

44. **Sheikh, M. A., and G. L. Taylor.** 2009. Crystal structure of the *Vibrio cholerae* ferric uptake regulator (Fur) reveals insights into metal co-ordination. Mol Microbiol **72:**1208-20.

45. **Skouloubris, S., A. Labigne, and H. De Reuse.** 2001. The AmiE aliphatic amidase and AmiF formamidase of *Helicobacter pylori*: natural evolution of two enzyme paralogues. Mol Microbiol **40:**596-609.

46. **Sreerama, N., and R. W. Woody.** 2000. Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. Anal Biochem **287:**252-60.

47. **Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee, and S. Falkow.** 2003. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. Infect Immun **71:**2643-55.

48. **Traore, D. A., A. El Ghazouani, S. Ilango, J. Dupuy, L. Jacquamet, J. L. Ferrer, C. Caux-Thang, V. Duarte, and J. M. Latour.** 2006. Crystal structure of the apo-PerR-Zn protein from *Bacillus subtilis*. Mol Microbiol **61:**1211-9.

49. **van Vliet, A. H., J. Stoof, S. W. Poppelaars, S. Bereswill, G. Homuth, M. Kist, E. J. Kuipers, and J. G. Kusters.** 2003. Differential regulation of amidase- and

formamidase-mediated ammonia production by the *Helicobacter pylori* Fur repressor. J Biol Chem **278:**9052-7.

50. **van Vliet, A. H., J. Stoof, R. Vlasblom, S. A. Wainwright, N. J. Hughes, D. J. Kelly, S. Bereswill, J. J. Bijlsma, T. Hoogenboezem, C. M. Vandenbroucke-Grauls, M. Kist, E. J. Kuipers, and J. G. Kusters.** 2002. The role of the Ferric Uptake Regulator (Fur) in regulation of *Helicobacter pylori* iron uptake. Helicobacter **7:**237-44.

51. **Vitale, S., C. Fauquant, D. Lascoux, K. Schauer, C. Saint-Pierre, and I. Michaud-Soret.** 2009. A ZnS(4) structural zinc site in the *Helicobacter pylori* ferric uptake regulator. Biochemistry **48:**5582-91.

Chapter Five

Discussion

Fur is a thoroughly studied regulatory protein found in a wide variety of Gram positive and Gram negative organisms, and yet what remains unknown about Fur poses a large volume of potential work. The goal of this thesis was to better understand Fur regulation in *Helicobacter pylori*, an organism that is not only medically important, but has also been shown to utilize Fur in a unique manner. The ability to use Fur as a repressor in the absence of its iron co-factor (*apo*-Fur) remains at present particular to *H. pylori*.

In the process of completing this thesis, we have shown that both iron-bound and *apo* Fur regulation of target genes can be monitored by GFP reporter plasmids in a manner that is comparable to more labor intensive procedures (e.g. RNase protection assays, Northern blots, etc.) (11). We believe that this type of genetic tool could be useful for screening a large pool of Fur mutants, and its creation led us to determine the importance of a single nucleotide within the *sodB* promoter Fur box for proper *apo*-Fur regulation of this gene. That study was the first time that a specific nucleotide residue was shown to be important for Fur regulation and in particular for Fur binding to the Fur box within the *sodB* promoter (10). These data suggest, at least for *apo*-Fur repression of *sodB*, that DNA binding sequences play a large role in Fur regulation (10). This being said, regulation is a complex process that not only involves the correct DNA binding sequence but also a functioning protein. Therefore, we also studied the role of specific amino acid residues in Fur functioning. These amino acids were selected based on their

conservation among organisms in which Fur has been well characterized and their overall importance for proper Fur function (9). Interestingly, while several of the conserved residues appear to be important in Fur from multiple species (e.g. E110), one residue that was important in *Vibrio cholerae* Fur function was not important for *H. pylori* Fur regulation (V64) (9). This implies that there are likely core amino acids that are necessary for proper functioning in all or nearly all Furs, but that other residues, even highly conserved ones are not functionally conserved. This is likely due to alterations in overall structure of Fur from one organism to the next; a conserved residue in one organism may be in a position to interact with DNA but in another organism that same residue may be buried and not in a position to interact with DNA. While these studies have shown that Fur regulation depends on both the DNA binding sequence and on the Fur amino acid sequence, there is clearly much more to be learned about this important regulatory protein.

Endogenous plasmids and H. pylori*: Unanswered questions*

It is estimated that approximately 50% of *H. pylori* isolates contain endogenous plasmids (33), and yet the purpose of these plasmids in the *H. pylori* life cycle is unclear. The plasmids do not carry antibiotic resistance markers, virulence factors or any genes that seem to provide a fitness advantage to the bacterium. However, half of *H. pylori* isolates will carry them. Why would the organism "waste" energy and resources to maintain plasmids that do not serve a helpful purpose? The answer must be that these cryptic plasmids do aid the bacteria in some as yet undescribed manner. While it is unclear what the role of the plasmids may be, genes commonly found on all of the

characterized plasmids share homology to the *mobA, mobB, mobC,* and/or *mobD* genes (11, 24-25), which are involved in DNA mobilization via conjugation. Thus, perhaps the plasmids play a role in promoting genetic diversity among *H. pylori* strains. Indeed, the first report of conjugation-like DNA transfer in *H. pylori* was made in 1998 (28), and the presence of *mob* genes on endogenous plasmids has subsequently been linked to this type of DNA transfer (24-25). This being said, there are many questions that remain about conjugation in *H. pylori*. For instance, it is not known how frequently this type of DNA transfer is used *in vivo* and what percentage of total gene transfer is the result of conjugation, as opposed to natural transformation, or how conjugation contributes to the pleomorphic nature of this organism. Additionally, the exact mechanism of conjugation has not yet been elucidated for *H. pylori*, and it is unclear if conjugation can take place in strains that do not contain endogenous plasmids. It has been suggested that the machinery for this method of DNA transfer is at least partially encoded on the chromosome (2). Overall, it is likely that in addition to the natural competence of *H. pylori*, the endogenous plasmids that contain *mob* genes contribute to the genetic diversity of this organism. Future studies should aim to help understand the basic mechanism and role of conjugation in *H. pylori.*

In addition to the contribution of endogenous plasmids to genetic diversity in *H. pylori*, there are several other aspects of plasmid biology that remain to be elucidated. For instance can *H. pylori* strains carry multiple plasmids or only one at a time? To the best of our knowledge, there has not been a report of *H. pylori* isolates that contain more than one plasmid. In fact, evidence exists to suggest that when a plasmid is transformed into a strain that carries an endogenous plasmid, the plasmids are recombined so that only

one plasmid is carried instead of two (B.M. Carpenter, K.R. Jones, and D.S. Merrell unpublished data). Studies could easily be designed to analyze this phenomenon. If the endogenous plasmid could be marked with an antibiotic resistance marker that differs from the plasmid to be transformed (non-endogenous plasmid), then replicate plating could be performed onto media containing no antibiotics, each antibiotic independently, and both antibiotics. Several colonies from each of these antibiotic selection plates could be isolated, and plasmid(s) harvested from these transformants. The plasmids could then be digested with restriction enzymes and sequenced to determine their relationship to the parental plasmids. For instance, a colony that grew on media containing both antibiotics could be carrying both plasmids or one recombined version that contains both antibiotic markers. A better understanding of how many different plasmids *H. pylori* can carry would influence the plasmid based genetic tools utilized in the study of this organism as well as influence the creation of new plasmid based genetic tools.

Since the pTM117 complementation and GFP reporter plasmid was described (11), another break though in *H. pylori* plasmid based genetic tools was reported – an inducible expression system (7). For the first time, conditional mutations can be constructed in essential genes in *H. pylori*. Additionally, this system could be used to determine optimal levels of expression of non-essential genes. Even though control of expression in this system is somewhat leaky (7), it could still be used to determine the level of *fur* expression that is needed in this organism. By placing *fur* expression under the control of an inducible promoter, the regulation of target genes could be monitored under various levels of *fur* expression and compared to regulation in wild-type bacteria. Data from these types of studies would help establish a range of *fur* expression necessary

for optimal regulation of its target genes. In addition, this system could be used to study what happens to the bacteria if Fur is over expressed or to determine if certain Fur mutations have a dominant negative effect on regulation. If this inducible plasmid was used in conjunction with pulse chase studies to determine the kinetics of Fur production, stability, and degradation, there is a real potential to determine just how much Fur is necessary for proper Fur regulation in *H. pylori*.

H. pylori *Fur box consensus sequences – does such a thing exist?*

Identification of a Fur box consensus sequence for both iron-bound and *apo*-Fur has proven to be difficult in *H. pylori*. This in part is due to the high A/T content of the *H. pylori* genome; saying that the Fur box consensus sequences are A/T rich (12) does not mean much for this organism. The 19bp Fur box consensus sequence from *Escherichia coli* (GATAATGATAATCATTATC) has long been considered to be the "gold standard" comparison for Fur boxes (16), but subsequent reevaluation of this sequence and the Fur box sequences in *Bacillus subtilis* suggest that the core Fur box is a 15bp, 7-1-7 repeat (TGATAATNATTATCA) (3). In comparison, there has been no good consensus sequence identified for the iron-bound Fur box in *H. pylori*; it was originally considered to be NNNNNAATAATNNTNANN (30). However, upon reexamination of the Fur boxes from known iron-bound Fur repressed genes in *H. pylori*, a new consensus sequence has recently been determined to be TAATAATNATTATTA, which represents a 15bp, 7-1-7 palindromic repeat (O.Q. Pich and D.S. Merrell unpublished data). This sequence differs from the sequence described above for *B. subtilis* and *E. coli* only at bases 2 and 14 and suggests that there are core bases that are

essential for Fur binding in several if not all organisms, which utilize Fur. Additionally, it is possible that these differences may impart species specific Fur/Fur box recognition.

Evaluation of Fur box sequences in *H. pylori* has been somewhat hindered by the fact that the Fur boxes often overlap the -10 and/or -35 promoter elements. This makes mutational studies of the promoters difficult since these elements are essential for gene expression. Therefore, a better approach might be to insert the Fur box sequences of known iron-bound repressed genes or constructed Fur boxes into the promoter of a reporter gene. In this manner, expression of the reporter would be dependent on the interaction between Fur and the Fur box. Mutations could then be made in the Fur boxes to determine the influence of each base on regulation. Also, electrophoretic mobility shift assays (EMSAs) could be performed on these Fur boxes (wild-type or mutant) to further understand the role of individual bases in Fur DNA interaction. Having a consensus sequence should also allow for the identification and study of potential new iron-bound Fur targets.

While there is now a putative conserved consensus sequence for iron-bound Fur in *H. pylori*, there is no such sequence for *apo*-Fur. It is known from our work on the *sodB* promoter that a single base within the Fur box region can drastically influence *apo*-Fur regulation of this gene. This seems to suggest that certain bases within the Fur box are more important than others for *apo*-Fur repression. However, with currently only two genes (*pfr* and *sodB*) shown to be regulated by Fur in this manner, it is hard to draw conclusions about what is necessary for *apo*-Fur to recognize a target gene's promoter. This difficulty is exacerbated by the overall lack of homology between the Fur boxes of *pfr* and *sodB* (12). In spite of this, it does appear that the sequence AAATGA is

important since it is conserved in both the *sodB* and *pfr* Fur boxes (O.Q. Pich and D.S. Merrell unpublished data). Given the limited number of *apo*-Fur repressed genes, there is a clear need for more genes to be shown to be regulated by *apo*-Fur through transcriptional analysis, DNase Footprinting, EMSAs, etc. Only then will there be enough predicted *apo*-Fur boxes to determine a putative *apo*-Fur box by sequence comparison alone. Additionally, it is quite possible that for *apo*-Fur repression, the actual sequence of the Fur box is less important than the overall conformation of the binding region. In other words, perhaps it is not the sequence of the DNA, but rather the shape of the DNA, that determines Fur binding. Clearly, studies like those described for determination of the iron-bound Fur box will need to be applied to help determine the *apo*-Fur box consensus sequence and to gain a better understanding of *apo*-Fur regulation in *H. pylori*.

The "black box" that is apo-*Fur regulation*

For as much as is known about Fur regulation in *H. pylori*, what remains unknown poses a large volume of potential work. This is especially true where *apo*-Fur regulation is concerned. Given that *apo*-Fur has only definitively been shown to function as a repressor in *H. pylori* and despite several studies that support its existence (12), the existence of this form of Fur regulation is still somewhat contentious. Debate aside, there are still several aspects of this type of regulation to be defined. For instance, how does *apo*-Fur compare to iron-bound Fur? It remains to be determined if *apo*-Fur functions as a monomer or a dimer. If as a dimer, does the structure differ from that of the iron-bound Fur dimer? One way to address this question may be to perform cross linking studies

with Fur in the presence of *apo*-Fur regulated promoter DNA under *apo* conditions. These reactions could be compared to *apo*-Fur alone and *apo*-Fur cross linking reactions run without the DNA. The size of the reaction products could determine if *apo*-Fur is interacting with target DNA as a monomer, dimer, or tetramer. We know from similar cross linking studies performed with Fur and the *amiE* promoter DNA under iron substitution conditions that the tetrameric form of the protein predominates (B.M. Carpenter and D.S. Merrell unpublished data), which agrees with what is known about iron-bound Fur interaction with DNA from other organisms (34). Quantitative size exclusion chromatography studies on purified *H. pylori* Fur suggest that in the absence of metal, Fur exists as a monomer (39). However, it could be possible that *apo*-Fur functions as a dimer but in the absence of target DNA the dimers are unstable, and thus *apo*-Fur appears as a monomer in the size exclusion experiments. Ideally, resolution of the crystal structure of *apo*-Fur bound to DNA could be determined to help address potential differences between the functional forms of *apo*-Fur and iron-bound Fur. Based on our understanding of how metal binding induces conformational changes in Fur, which allows the protein to dimerize and bind DNA, it is very likely that *apo*-Fur is functioning as a monomer or a dimer of an overall different conformation.

As mentioned, to date the use of *apo*-Fur as a repressor is unique to *H. pylori*. This being said, there are microarray analyses that suggest that *apo*-Fur regulation may be occurring in *Campylobacter jejuni* (26) and *Desulfovibrio vulgaris* Hildenborough (4); however, no follow-up studies that look at DNA binding or DNase footprinting have been performed on Fur and the genes predicted to be *apo*-Fur targets from these organisms. Until those studies have been conducted, there is no way to know for certain if *apo*-Fur is

directly repressing the predicted target genes or whether changes in expression are the result of indirect Fur regulation. A recent study that asked whether heterologous Fur proteins from various species could complement a Δ*fur H. pylori* mutation showed that the Fur proteins from *E. coli*, *V. cholerae*, *C. jejuni*, *Pseudomonas aeruginosa*, and *D. vulgaris* Hildenborough were not able to complement *apo*-Fur regulation (31). This lack of complementation suggests that *apo*-Fur repression is limited to *H. pylori*. However, it should be noted that *apo*-Fur regulation has not yet been assessed in other species within the *Helicobacter* genus or in other closely related bacteria like the *Wolinella* species.

There are 17 genes predicted to be in the *apo*-Fur regulon (19), and yet only two of these genes have been shown to be directly repressed by *apo*-Fur through DNase footprinting and/or DNA binding studies. As mentioned above, the limited number of confirmed targets for *apo*-Fur has greatly hindered our ability to define an *apo*-Fur box and to better understand the role *apo*-Fur plays within the bacterial lifecycle. It is therefore crucial that more of the predicted *apo*-Fur target genes be studied to determine if they are in fact regulated as predicted. While this may be somewhat of a large undertaking, the potential expansion of our understanding of *apo*-Fur would make it a worthwhile endeavor.

A classic repressor in a not so classic organism

Although Fur is arguably one of the best characterized bacterial regulatory proteins, in *H. pylori* there are several aspects of this protein that are still not well understood. For instance, how does the structure of *H. pylori* Fur compare to that of *P. aeruginosa* and *V. cholerae*, organisms for which the Fur crystal structure has been

resolved? We know from a recent study that the *H. pylori* Fur structural Zn^{2+} molecule is coordinated by two CXXC motifs (39). Neither of the crystalized Furs coordinate their structural metal molecule through Cs (34-35). Comparatively, the use CXXC motifs to coordinate a metal ion makes Fur in *H. pylori* more similar to that of *E. coli* (14) or to *B. subtilis* PerR, which is in the Fur family of metalloproteins (37). Thus, the question arises as to how this metal coordination alters the overall structure of *H. pylori* Fur and in particular the structure of the C-terminal domain of the protein, which is involved in not only metal binding but also dimerization. In addition, the N-terminal domain of *H. pylori* Fur carries an amino acid extension as compared to that of *E. coli*, *P. aeruginosa*, and *V. cholerae*. There are approximately ten extra amino acid residues, and this N-terminal extension is conserved only among closely related ε-Proteobacteria (9). How does having these extra amino acids in the DNA binding region of the protein change its overall interaction with target DNA? Is the extension important for Fur function? Are these residues important for *apo*-Fur function? It is interesting that in C. *jejuni*, microarray analysis of the Fur regulon suggests that *apo*-Fur regulation may be occurring in this organism (26), and like *H. pylori* Fur, *C. jejuni* Fur carries an N-terminal extension. One way to investigate the role of these residues in the absence of a crystal structure, would be to construct mutant Fur proteins where some or all of these "extra" N-terminal amino acid residues are deleted. These mutants could then be assessed for their ability to regulate target genes *in vivo* and to bind DNA *in vitro*. If these residues are shown to be important for Fur regulation, especially *apo* regulation, then these residues could be added to Fur from an organism, like *E. coli*, that lacks an N-terminal extension

and shows no evidence of *apo*-Fur regulation to determine if the functional capability of *apo*-Fur could be gained through the addition of those residues.

Given that *in vitro E. coli* Fur has been shown to bind several divalent metal ions in addition to iron (23) and since Zn^{2+} and Mn^{2+} are routinely used in *in vitro* analyses as substitutes for Fe^{2+}, is it possible that under severe iron limitation that Fur may utilize other metal ions to preserve regulation? It has been suggested that *H. pylori* Fur functions as a rheostat for iron availability in the bacterial cell (18). Certainly, in an organism like *H. pylori* that has relatively few regulatory proteins, the bacterium may have adapted to preserve some aspects of Fur regulation even under severely iron limited conditions. To determine if there is any biological relevance to the *in vitro* abilities of Fur to bind other metal ions, bacterial cells could be exposed to metal chelators, and then after a period of time, metals of interest could be added back to the culture. Next, the restoration of Fur regulation could be assessed through monitoring of iron-bound and *apo* regulated targets through the use of RNase protection assays, Northern blots, or transcriptional reporters. If Fur regulation could be partially or fully restored in the absence of iron and in the presence of another divalent metal, this would indicate that *H. pylori* has evolved to utilize other available metal ions under extreme conditions. Additionally, equilibrium dialysis and atomic absorption spectroscopy could be performed on purified *H. pylori* Fur dialyzed against various metal ion baths to determine the affinity of Fur for each different metal. While iron is the biologically relevant metal co-factor (32), in its absence, Fur may be able to utilize other metals.

While *fur* is not an essential gene in *H. pylori* (13), it does appear to provide an early colonization advantage in the host (8, 22). How does having Fur help the bacteria

establish infection? Is it simply that Fur allows for proper uptake and storage of iron, a critical nutrient? Likely this is not just the case. Fur is involved in many aspects of homeostasis that are not clearly linked to iron uptake (12). These roles include regulation of *sodB*, which affects oxidative stress (10, 20), and Fur's involvement in mediating acid related stress through *amiE* regulation (11, 38). Interestingly, a recent study showed that regulation of the entire urease gene cluster is down regulated in a *fur* mutant (29); therefore, having an intact Fur could help combat the pH stress that the bacteria faces within the gastric environment not only through *amiE* but also through urease.

In addition to animal model infection studies with wildtype (WT) and *fur* mutant strains to examine pathology, colonization rates, etc., if sufficient quantities of purified material could be obtained, RNA could be isolated from the stomachs of animals infected with WT and *fur* mutant *H. pylori* and microarray analysis could be performed on this RNA. This type of study would present a global picture of gene transcription *in vivo* with and without an intact *fur* gene. Also, if RNA was isolated over several days post infection, then changes in the importance of Fur regulation could be assessed over time. These studies would represent a starting point for further exploration of the role of Fur in colonization as more definitive experiments would be needed to confirm any of the trends shown by microarray. Overall, we predict that it is likely that many genes in the Fur regulon contribute to increasing the colonization efficiency of *H. pylori* and that multiple members or the whole of the regulon enhances *H. pylori* fitness.

The curious case of Fur activation in H. pylori

In addition to functioning as a repressor in both its iron-bound and *apo* forms, *H. pylori* Fur has also been shown to function as a transcriptional activator in both of these forms. Despite there being several genes predicted by microarray analysis to be Fur induced (15, 19), the proven examples of these activated genes are few - *fur* autoregulation utilizes *apo*-Fur activation (18) and *nifS* has been shown to be activated by iron-bound Fur (1). One trend in Fur activation among bacterial species including *H. pylori* is that the Fur boxes are located further upstream beyond the -10 and -35 promoter elements within the target promoter (12). However, what is not understood is how Fur binding at these Fur boxes results in activation. It could be that Fur binding alters the shape of the DNA helix in such a way that binding of the RNA polymerase is facilitated. Alternatively, Fur could be working in concert with another "Up" element; together they would enhance the binding of RNA polymerase. Co-immunoprecipitation studies could be performed to help determine if Fur is complexing with another protein in the activation process. It is also possible that when Fur binds to an activation site, that this binding dislodges a negative regulator that blocks RNA polymerase from binding; therefore, by removing the negative regulator, transcription can begin. Having a better understanding of how the Fur-activated genes are repressed would help to determine how Fur is acting as an activator. This being said, in order to really understand Fur activation, more of the predicted targets needs to be shown to directly be activated by Fur. This would encompass the types of studies as described for *apo*-Fur targets - transcriptional analysis (Northern blots, RPAs), EMSAs and/or DNase Footprinting. In addition, since there is no consensus sequence for an activation Fur box, primer extension studies may

be necessary to determine the transcriptional start sites of these genes so as to better predict the Fur box location. Also, what differentiates an *apo*-Fur activation Fur box from an iron-bound activation Fur box remains to be elucidated. Perhaps which form of Fur binds is determined by something other than binding sequence like the overall shape of the DNA.

What is also not well understood about Fur activation is what amino acid residues are important for this type of Fur regulation. Are the residues that were found to be important for iron-bound and *apo*-Fur repression also important for iron-bound and *apo*-Fur activation, respectively, or are entirely different residues important for activation? The Fur mutant proteins characterized in Chapter Four for both types of Fur repression could be used in similar studies with Fur activation targets to help elucidate the residues that are critical for activation. However, given that the only *apo*-Fur activation target that has been studied is *fur* itself and *fur* autoregulation is a complex mixture of iron-bound repression and *apo* activation (17-18), it would likely be necessary to use another *apo*-Fur activated gene for these studies. Additionally, it is currently not known whether iron-bound Fur activation requires dimer formation or whether *apo*-Fur activation is mediated through Fur monomers or dimers. Clearly, there is much to be learned about Fur activation in *H. pylori*, and through a better understanding of this type of Fur regulation in this organism, we will gain insight into how Fur activation functions in other bacteria.

The future of Fur

Beyond increasing our understanding of the role Fur plays in bacterial gene regulation in *H. pylori* where Fur uniquely functions as a repressor in its *apo* form, studying Fur can have broader implications for the relationship between bacterial pathogens and their host. In a recent study, a real-time PCR assay was developed to determine if fleas were infected with *Yersinia pestis* using primers specific for *fur* (21). It was reported that as few as 300 bacteria were needed in infected fleas in order to be detected in this assay (21). This is the first time that Fur has been used as a marker for bacterial infection, and opens the door for its use in identifying other infections. Given that Fur is utilized by a wide variety of Gram negative and Gram positive bacteria (12), the potential applications for this type of assay are quite large. A PCR based assay could be used to make a preliminary diagnosis while waiting for confirmation through cultures. One possible drawback to using *fur* as a diagnostic tool is that there is significant homology among *fur* genes, so considerable care would need to be taken in designing primers for these assays. Interestingly, this technology would not be limited to identifying human pathogens but could just as easily be used in identifying animal pathogens. For instance, such assays could be used to identify outbreaks of *Edwardsiella tarda*, which is known to express *fur* (36), in aquaculture facilities or even to monitor for the presence of this pathogen within the fish population. Likewise, plant pathogens also express *fur* genes, so this type of assay could be used to identify infections in crops or nurseries. The use of *fur* as a marker of bacterial infections is potentially a viable addition to our repertoire of diagnostic techniques.

It was shown several years ago that *H. pylori* Fur is important for not only proper iron homeostasis (5) but also for acid resistance (6, 22, 29). In addition to its role in these two aspects of bacterial homeostasis and adaptation to environmental stress, Fur has been shown to be involved in nitrogen metabolism (38) and in the response to oxidative stress (10, 20). Because of its broad involvement in adaptation and colonization (8, 22) and given the fact that antibiotic resistance had become a critical issue in treating *H. pylori* infections (27), Fur makes an interesting potential antibiotic or treatment target. Because *fur* is not essential in *H. pylori* (13), an antibiotic that only targets this gene or its product could not be used by itself as elimination of *fur* does not result in death of the organism. However, it could be that by targeting *fur*/Fur the susceptibility of *H. pylori* to other antibiotics would increase. It would be necessary to show this prior to investing time and money in designing a drug to target this regulatory protein. To determine if a *fur* mutant strain of *H. pylori* is more susceptible to antibiotics there are several simple experiments that could be conducted. First, disc diffusion experiments could be performed on bacteria grown in agar plates and the zones of inhibition could be compared between the two strains. The strains could also be grown in liquid cultures containing various amount of antibiotic and then plated for single colonies to determine the percent survival of each strain under the various concentrations of antibiotics as compared to no antibiotic controls. This analysis would allow for the determination of minimum inhibitory concentrations (MICs) and/or minimum bactericidal concentrations (MBCs) for WT and the *fur* mutant. Additionally, kill curves for the WT and *fur* mutant strains could be generated by temporal plating of strains grow in culture media containing the antibiotic. These would be compared to the number of colony forming units (CFUs) for each strain

in the absence of antibiotics. If the *fur* mutant is more susceptible to antibiotics than the WT, we would expect to see larger zones of inhibition on the disc diffusion plates, a decrease in the MIC and/or MBC, and a sharper declining slope on the kill curves as compared to the WT strain. These studies should be conducted using antibiotics like clarithromycin, amoxicillin, and metronidazole that are commonly used in first line anti-*H. pylori* therapy and those like levofloxacin and doxycycline now used as second line therapies (27).

Once it was established that the absence of *fur* makes *H. pylori* more susceptible to antibiotic treatment, development of drugs to target *fur* or its gene product could begin. Some possible designs could be a small inhibitory RNA (siRNA) that targets and prevents *fur* transcription or a small molecule that mimics a Fur box and upon uptake into the bacterial cell would irreversibly bind to Fur. Alternatively, a protein that complexes with Fur and titrates Fur from the bacterial cell's protein pool could be developed. While the development of an antibiotic or treatment that targets Fur specifically would require a large investment of time, money and efforts, it may be necessary if no other new antibiotics are developed and if as predicted, resistance rates to current antibiotics continue to rise.

Conclusions

H. pylori is a unique human pathogen that in the 30 years since its discovery has revolutionized our understanding of the origins of gastric maladies. However, there are still some questions about the basic biology of *H. pylori* that have not been answered. These include those discussed above for endogenous *H. pylori* plasmids. Furthermore,

while Fur is a classic and well studied regulatory protein, which is utilized by a wide variety of bacterial species, the fine tuning and adaptation of this protein in *H. pylori* leaves us with many unanswered questions regarding the role of Fur in *H. pylori* as well as in the larger realm of bacteria. Fur is utilized as an activator and repressor in both its iron-bound and *apo* forms - something that had not been shown for any other organism. Given the uniqueness of Fur regulation in *H. pylori*, there is clearly much to be learned about Fur in this pathogen. We have limited understanding of *apo*-Fur repression and Fur activation in terms of protein structure, gene targets, and protein-DNA interaction, and it appears from amino acid sequence comparison that the functional conformation of Fur may be different than that of *P. aeruginosa* and *V. cholerae*. This is likely especially true in the N-terminal portion of the protein. The potential use of Fur as a diagnostic marker for infection and as a drug target necessitates the continued study of this regulatory protein not only in *H. pylori* but in other bacteria as well.

References

1. **Alamuri, P., N. Mehta, A. Burk, and R. J. Maier.** 2006. Regulation of the *Helicobacter pylori* Fe-S cluster synthesis protein NifS by iron, oxidative stress conditions, and Fur. J Bacteriol **188:**5325-30.

2. **Backert, S., T. Kwok, and W. Konig.** 2005. Conjugative plasmid DNA transfer in *Helicobacter pylori* mediated by chromosomally encoded relaxase and TraG-like proteins. Microbiology **151:**3493-503.

3. **Baichoo, N., and J. D. Helmann.** 2002. Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. J Bacteriol **184:**5826-32.

4. **Bender, K. S., H. C. Yen, C. L. Hemme, Z. Yang, Z. He, Q. He, J. Zhou, K. H. Huang, E. J. Alm, T. C. Hazen, A. P. Arkin, and J. D. Wall.** 2007. Analysis of a ferric uptake regulator (Fur) mutant of *Desulfovibrio vulgaris* Hildenborough. Appl Environ Microbiol **73:**5389-400.

5. **Bereswill, S., S. Greiner, A. H. van Vliet, B. Waidner, F. Fassbinder, E. Schiltz, J. G. Kusters, and M. Kist.** 2000. Regulation of ferritin-mediated cytoplasmic iron storage by the ferric uptake regulator homolog (Fur) of *Helicobacter pylori*. J Bacteriol **182:**5948-53.

6. **Bijlsma, J. J., B. Waidner, A. H. Vliet, N. J. Hughes, S. Hag, S. Bereswill, D. J. Kelly, C. M. Vandenbroucke-Grauls, M. Kist, and J. G. Kusters.** 2002. The *Helicobacter pylori* homologue of the Ferric uptake regulator is involved in acid resistance. Infect Immun **70:**606-11.

7. **Boneca, I. G., C. Ecobichon, C. Chaput, A. Mathieu, S. Guadagnini, M. C. Prevost, F. Colland, A. Labigne, and H. de Reuse.** 2008. Development of inducible systems to engineer conditional mutants of essential genes of *Helicobacter pylori*. Appl Environ Microbiol **74:**2095-102.

8. **Bury-Mone, S., J. M. Thiberge, M. Contreras, A. Maitournam, A. Labigne, and H. De Reuse.** 2004. Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. Mol Microbiol **53:**623-38.

9. **Carpenter, B. M., H. Gancz, S. L. Benoit, S. Evans, S. L. J. Michel, R. J. Maier, and D. S. Merrell.** 2010. Mutagenesis of Conserved Amino Acids of *Helicobacter pylori* Fur Reveals Residues Important for Function. J Bacteriol ***submitted***.

10. **Carpenter, B. M., H. Gancz, R. P. Gonzalez-Nieves, A. L. West, J. M. Whitmire, S. L. Michel, and D. S. Merrell.** 2009. A single nucleotide change affects Fur-dependent regulation of *sodB* in *H. pylori*. PLoS One **4:**e5369.

11. **Carpenter, B. M., T. K. McDaniel, J. M. Whitmire, H. Gancz, S. Guidotti, S. Censini, and D. S. Merrell.** 2007. Expanding the *Helicobacter pylori* genetic toolbox: modification of an endogenous plasmid for use as a transcriptional reporter and complementation vector. Appl Environ Microbiol **73:**7506-14.

12. **Carpenter, B. M., J. M. Whitmire, and D. S. Merrell.** 2009. This is not your mother's repressor: the complex role of Fur in pathogenesis. Infect Immun **77:**2590-601.

13. **Chalker, A. F., H. W. Minehart, N. J. Hughes, K. K. Koretke, M. A. Lonetto, K. K. Brinkman, P. V. Warren, A. Lupas, M. J. Stanhope, J. R. Brown, and P. S. Hoffman.** 2001. Systematic identification of selective essential genes in *Helicobacter pylori* by genome prioritization and allelic replacement mutagenesis. J Bacteriol **183:**1259-68.

14. **Coy, M., C. Doyle, J. Besser, and J. B. Neilands.** 1994. Site-directed mutagenesis of the Ferric uptake regulation gene of *Escherichia coli*. Biometals **7:**292-8.

15. **Danielli, A., D. Roncarati, I. Delany, V. Chiarini, R. Rappuoli, and V. Scarlato.** 2006. In vivo dissection of the *Helicobacter pylori* Fur regulatory circuit by genome-wide location analysis. J Bacteriol **188:**4654-62.

16. **de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (Fur) repressor. J Bacteriol **169:**2624-30.

17. **Delany, I., G. Spohn, A. B. Pacheco, R. Ieva, C. Alaimo, R. Rappuoli, and V. Scarlato.** 2002. Autoregulation of *Helicobacter pylori* Fur revealed by functional analysis of the iron-binding site. Mol Microbiol **46:**1107-22.

18. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2003. An anti-repression Fur operator upstream of the promoter is required for iron-mediated transcriptional autoregulation in *Helicobacter pylori*. Mol Microbiol **50:**1329-38.

19. **Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. van Vliet, and G. Homuth.** 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. Microbiology **151:**533-46.

20. **Ernst, F. D., G. Homuth, J. Stoof, U. Mader, B. Waidner, E. J. Kuipers, M. Kist, J. G. Kusters, S. Bereswill, and A. H. van Vliet.** 2005. Iron-responsive regulation of the *Helicobacter pylori* iron-cofactored superoxide dismutase SodB is mediated by Fur. J Bacteriol **187:**3687-92.

21. **Gabitzsch, E. S., R. Vera-Tudela, R. J. Eisen, S. W. Bearden, K. L. Gage, and N. S. Zeidner.** 2008. Development of a real-time quantitative PCR assay to enumerate *Yersinia pestis* in fleas. Am J Trop Med Hyg **79:**99-101.

22. **Gancz, H., S. Censini, and D. S. Merrell.** 2006. Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen *Helicobacter pylori*. Infect Immun **74:**602-14.

23. **Hamed, M. Y.** 1993. Binding of the Ferric uptake regulation repressor protein (Fur) to Mn(II), Fe(II), Co(II), and Cu(II) ions as co-repressors: electronic absorption, equilibrium, and 57Fe Mossbauer studies. J Inorg Biochem **50:**193-210.

24. **Hofler, C., W. Fischer, D. Hofreuter, and R. Haas.** 2004. Cryptic plasmids in *Helicobacter pylori*: putative functions in conjugative transfer and microcin production. Int J Med Microbiol **294:**141-8.

25. **Hofreuter, D., and R. Haas.** 2002. Characterization of two cryptic *Helicobacter pylori* plasmids: a putative source for horizontal gene transfer and gene shuffling. J Bacteriol **184:**2755-66.

26. **Holmes, K., F. Mulholland, B. M. Pearson, C. Pin, J. McNicholl-Kennedy, J. M. Ketley, and J. M. Wells.** 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology **151:**243-57.

27. **Jones, K. R., J. H. Cha, and D. S. Merrell.** 2008. Who's Winning the War? Molecular Mechanisms of Antibiotic Resistance in *Helicobacter pylori*. Curr Drug Ther **3:**190-203.

28. **Kuipers, E. J., D. A. Israel, J. G. Kusters, and M. J. Blaser.** 1998. Evidence for a conjugation-like mechanism of DNA transfer in *Helicobacter pylori*. J Bacteriol **180:**2901-5.

29. **Lee, J. S., Y. H. Choe, J. H. Lee, H. J. Lee, and Y. O. Choi.** 2010. *Helicobacter pylori* urease activity is influenced by Ferric uptake regulator. Yonsei Med J **51:**39-44.

30. **Merrell, D. S., L. J. Thompson, C. C. Kim, H. Mitchell, L. S. Tompkins, A. Lee, and S. Falkow.** 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. Infect Immun **71:**6510-25.

31. **Miles, S., B.M. Carpenter, H. Gancz, D.S. Merrell.** 2010. *H. pylori apo*-Fur Regulation Appears Unconserved Across Species. J Micro ***in press***.

32. **Mills, S. A., and M. A. Marletta.** 2005. Metal binding characteristics and role of iron oxidation in the ferric uptake regulator from *Escherichia coli*. Biochemistry **44:**13553-9.

33. **Penfold, S. S., A. J. Lastovica, and B. G. Elisha.** 1988. Demonstration of plasmids in *Campylobacter pylori*. J Infect Dis **157:**850-1.

34. **Pohl, E., J. C. Haller, A. Mijovilovich, W. Meyer-Klaucke, E. Garman, and M. L. Vasil.** 2003. Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the Ferric uptake regulator. Mol Microbiol **47:**903-15.

35. **Sheikh, M. A., and G. L. Taylor.** 2009. Crystal structure of the *Vibrio cholerae* Ferric uptake regulator (Fur) reveals insights into metal co-ordination. Mol Microbiol **72:**1208-20.

36. **Sun, K., S. Cheng, F. Wang, and L. Sun.** 2009. Domain analysis of the *Edwardsiella tarda* ferric uptake regulator. J Gen Appl Microbiol **55:**351-8.

37. **Traore, D. A., A. El Ghazouani, S. Ilango, J. Dupuy, L. Jacquamet, J. L. Ferrer, C. Caux-Thang, V. Duarte, and J. M. Latour.** 2006. Crystal structure of the apo-PerR-Zn protein from *Bacillus subtilis*. Mol Microbiol **61:**1211-9.

38. **van Vliet, A. H., J. Stoof, S. W. Poppelaars, S. Bereswill, G. Homuth, M. Kist, E. J. Kuipers, and J. G. Kusters.** 2003. Differential regulation of amidase- and formamidase-mediated ammonia production by the *Helicobacter pylori* Fur repressor. J Biol Chem **278:**9052-7.

39. **Vitale, S., C. Fauquant, D. Lascoux, K. Schauer, C. Saint-Pierre, and I. Michaud-Soret.** 2009. A ZnS(4) structural zinc site in the *Helicobacter pylori* Ferric uptake regulator. Biochemistry **48:**5582-91.